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Analysis of ErbB2- and Growth Factor-Dependent Gene Expression in Breast Cells

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ABSTRACT

The ErbB family of growth factor receptors are important mediators of cellular signalling that control changes in an array of cellular responses such as proliferation, differentiation, migration, adhesion and apoptosis through the activation of various downstream signalling pathways. The ErbB family of receptor tyrosine kinases includes four members; EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. ErbB2 is an important target for breast cancer therapy because it is overexpressed by gene amplification in 25 to 30% of all breast cancers and was the target of the first successful therapeutic monoclonal antibody. Because of its clinical importance, understanding the role of ErbB2 and other members of the ErbB receptor family in cancer has been the subject of intense research. However, although much is now known about the signalling pathways activated downstream of the ErbB family members, the mechanisms involved in ErbB2-mediated tumourigenesis are still poorly understood.

The purpose of this study was to understand the underlying changes associated with ErbB2-dependent signalling and transformation. The major part of this study was focused on deciphering global changes in gene expression associated with ErbB2 overexpression and was analysed by microarray technology using a model human mammary luminal epithelial cell system. In addition, differential gene expression associated with downstream signalling events activated by two ErbB-specific ligands, EGF and HRG, were examined. Microarray analysis allowed the identification of potential molecular markers of ErbB2 overexpression, some of which have been previously implicated in breast or other types of cancer. Gene expression changes for a number of genes were validated by quantitative real-time PCR, and a number of genes were further validated at the protein level. Subsequently, members of a growth-regulatory signal transduction pathway involving interferon signalling found to have altered gene expression due to ErbB2 overexpression were further characterised, and their connection with ErbB2-depedent signalling and transformation investigated.

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ABBREVIATIONS

Chemicals and reagents

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
BSA	bovine serum albumin
CHAPS	(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)
CHX	cyclohexamide
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FCS	foetal calf serum
HCL	hydrogen chloride
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
NP-40	nonidet P-40
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TBS	tris buffered saline
Tris	tris-(hydroxymethyl)aminomethane

General

1DE	one-dimensional gel electrophoresis
2DE	two-dimensional gel electrophoresis
Ab	antibody (mAb: monoclonal Ab; pAb: polyclonal Ab)
ATP	adenosine triphosphate
Cdk	cyclin dependent kinase
cDNA	complimentary DNA
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence

ECM	extracellular matrix
EST	expressed sequence tag
FACS	fluorescence activated cell sorter
FDR	false discovery rate
FITC	fluorescein isothiocyanate
GAS	IFN gamma-activated site
GATE	IFN gamma-activated transcriptional element
GTP	guanosine trisphosphate
HMLEC	human mammary luminal epithelial cell
IP	immunoprecipitation
ISRE	IFN alpha-stimulated response element
kDa	kilodalton
LOWESS	locally weighted regression scatterplot smoother
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PH	pleckstrin homology domain
PTB	phosphor-tyrosine binding domain
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
SAM	significance analysis of microarrays
TMA	tissue microarrays
TRITC	tetramethylrhodamine isothiocyanate

Proteins, enzymes, growth factors and inhibitors

AREG	amphiregulin
BRCA1/2	breast cancer 1/2- early onset
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
ErbB2	erythroblastosis B-2
ErbB3	erythroblastosis B-3
ErbB4	erythroblastosis B-4

ERK	extracellular signal regulated kinase
FGF	fibroblast growth factor
GAP	GTPase activating protein
GRB2	growth factor receptor-bound protein 2
HGF	hepatocyte growth factor
HRG	heregulinβ1
IFN	interferon
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IRF	interferon regulatory factor
ISG	interferon stimulated genes
ISGF3	interferon stimulated gene factor 3
JAK	Janus kinase
JNK	c-Jun amino-terminal kinases
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
NGF	nerve growth factor
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositide phosphate
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Ras	retrovirus-associated DNA sequence
pRb	retinoblastoma protein
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase
SH2	src homology-2 domain
SH3	src homology-3 domain
STAT	signal transducers and activators of transcription
SV40	simian virus 40
TGF–α	transforming growth factor α
VEGF	vascular endothelial growth factor

Chapter 1: INTRODUCTION

1.1 Protein Tyrosine Kinases & Regulation of Cellular Life

Multicellular organisms live in a complex environment, and in order to thrive they must adapt to their surrounding through careful regulation of fundamental processes at the cellular level. Cells must be able to interact with each other, correctly interpret received signals and elicit an appropriate response in order to perform diverse physiological functions. Such signals are emitted and/or received through direct cell-cell interaction or through secreted molecules. Protein kinases play a key regulatory role in interpreting such signals in nearly all aspects of cell biology. They regulate processes such as metabolism, cell cycle progression, apoptosis, differentiation, development, immune system responses, nervous system function, cell movement and transcription.

The first observation to shed light into the possible involvement of protein kinases in the regulation of cellular functions was made in 1954, when a liver protein was found to transfer a phosphate group onto the protein casein (Burnett & Kennedy 1954). A year later, the role of protein phosphorylation became even more interesting when it was shown that the metabolic enzyme glycogen phosphorylase was regulated by addition and removal of a phosphate, suggesting that reversible phosphorylation could control enzyme activity (Fisher & Krebs 1955). Thus, protein phosphorylation began to emerge as a key regulator of cellular behaviour. Since then, considerable effort has been made to determine the physiological and pathological functions of protein kinases and their role in signal transduction.

The reversible protein phosphorylation catalyzed by kinases and phosphatases is now known to be the principal mechanism by which intracellular functions are regulated by extracellular signals (Cohen 2001). Based on the nature of the phosphorylated protein, kinases can be classified as protein serine/threonine kinases or protein tyrosine kinases. About 1.7% of all genes in the entire human genome are thought to encode for protein kinases, representing a total number of 518 proteins (Manning *et al.* 2002). This "kinome" consists of 385 serine/threonine (Ser/Thr) kinases, 90 protein-tyrosine (Tyr) kinases, and 43 tyrosine kinase-like proteins. Of the 90 protein-tyrosine kinases, 58 are receptors and 32 are non-receptor kinases. In addition, there is a group of enzymes that can catalyze the

phosphorylation of both threonine and tyrosine on target proteins, so called dual-specificity kinases (Robinson *et al.* 2000). The ratio of protein phosphoserine and phosphothreonine to phosphotyrosine in normal animal cells is about 3000/1 (Hunter & Sefton 1980). Despite this, protein tyrosine phosphorylation plays a crucial role in signal transduction. Typically, protein tyrosine kinases (PTKs) undergo auto-phosphorylation and phosphotyrosines can serve as docking sites for molecules that transmit downstream signals, which often include the activation of protein-Ser/Thr kinases. While a specific Ser/Thr kinase can phosphorylate many substrate molecules, Tyr kinases are not usually associated with such amplification. (Roskoski, Jr. 2004).

1.1.1 Receptor tyrosine kinases (RTKs)

A large group of human genes encode for proteins that function as membrane spanning cell surface receptors. These receptors can be classified into distinct families based upon the ligands they recognize, the biological response they induce, and their primary structures. One large family of such cell surface receptors are the receptor tyrosine kinases (RTKs). Their activation is induced by ligand binding, which leads to receptor dimerization resulting in auto-phosphorylation and cross-phosphorylation of their cytoplasmic domains. This tyrosine phosphorylation is crucial for the recruitment and activation of a variety of signalling proteins containing SH2 (Src-homology 2), SH3 (Src homology 3), and PTB (phosphotyrosine binding) domains. These proteins in turn activate downstream signalling pathways, which include changes in enzyme activity and protein stability, that ultimately result in induction or repression of gene expression, thereby eliciting specific cellular responses (Schlessinger 2000). The major events governing signal transduction involve signal cascades of protein-protein or protein-lipid interactions often achieved through a series of consecutive phosphorylation events. The importance of ligand-induced intracellular tyrosine phosphorylation as a means of transmembrane signal transduction can be further highlighted by the ability of receptors lacking intrinsic catalytic activity, such as the cytokine receptors, to form noncovalent interactions with non-receptor tyrosine kinases (e.g. Src-family and JAKs). These PTK-receptor complexes can then activate downstream molecules to propagate the signals (Taniguchi 1995).

The first receptor to be recognized as a tyrosine-specific protein kinase was the receptor for epidermal growth factor (EGF) (Carpenter *et al.* 1979). Many other receptors for growth factors are also receptor tyrosine kinases. These include receptors for platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), insulin, insulin-like growth factor-1 (IGF-1), nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and macrophage colony stimulating factor (M-CSF). These RTKs play a role in the control of the most fundamental cellular processes, and their deregulation occurs in a variety of diseases including cancer and diabetes, as well as in autoimmune, cardiovascular, inflammatory and nervous disorders. Not surprisingly, there is a great deal of interest in elucidating the intricate mechanisms of action of these kinases in order to implement targeted and effective therapies to these common human pathologies.

1.1.2 Tyrosine kinases & cancer

Cellular proliferation is a highly regulated process. Regulation of a cell's proliferative capacity is of critical importance, and errors in such control can result in the development of diseases such as cancer. It is now established that cancer arises as a consequence of successive genetic alterations that drive the progressive transformation of normal cells into cancerous cells. Through this process, these malignant cells acquire a proliferative advantage and rapidly outgrow normal, healthy cells. In this context, Hanahan and Weinberg (Hanahan & Weinberg 2000) have reviewed the mechanisms of cancer development whereby specific changes in cell behaviour will render them capable of breaching the normal anti-cancer defence mechanisms present in cells and tissues. These "hallmarks" of cancer consist of (i) self-sufficiency in growth signals; (ii) insensitivity of cells to growth-inhibitory signals; (iii) capability to evade programmed cell death; (iv) limitless replicative potential; (v) increased angiogenesis; and (vi) ability to invade tissues and metastasize.

RTK signalling functions directly relate to these hallmarks of cancer. RTKs are essential mediators of growth-regulatory signals, and as such can determine the fate of cells by interpreting regulatory messages, activating intracellular signalling pathways and ultimately altering gene expression and protein turnover. The first evidence to support the role of RTKs in transformation was provided by the

demonstration that EGFR is the cellular homolog of the avian erythroblastosis virus v-*erb*B oncogene (Downward *et al.* 1984). This oncogene encodes an N-terminal-truncated form of *erb*B1 (EGFR) which lacks the extracellular domain and exhibits several intracellular mutations, resulting in ligand-independent dimerization, phosphorylation and constitutive signalling. Since then, gain-of-function mutations, deletions, chromosomal translocations, autocrine-paracrine stimulation by overexpression of ligands, retroviral transduction of protooncogenes and gene amplification resulting in PTK overexpression have all been shown to be mechanisms by which PTKs can induce oncogenic transformation (Madhusudan & Ganesan 2004). Table 1.1 shows examples of PTKs involved in human cancers and reinforces the fact that the evidence for their involvement in cancer is overwhelming. As a consequence, protein tyrosine kinases have become the most important family of proteins in the search for targeted cancer therapies.

Inhibition of activated PTKs through small molecules or antibody-based strategies has emerged as an effective approach to cancer therapy. Gleevec (Novartis Pharmaceutical), a small molecule that reversibly competes with ATP for binding to the kinase domain of PDGFR, c-Kit, and Abl, has shown remarkable activity in CML (chronic myelogenous leukemia) and GIST (gastrointestinal stromal tumour) patients (Arteaga & Baselga 2004). The VEGFR-2 inhibitor Semaxanib (SUGEN/ Pharmacia) competitively blocks ATP binding to the tyrosine kinase domain of VEGFR-2, thereby inhibiting tumour vascularization and growth of multiple tumour types (Fong *et al.* 1999). Inhibitors targeted specifically to the EGFR receptor family (discussed below) have also been shown to induce positive responses in patients with breast and a subset of lung cancers, further demonstrating the utility of targeting the PTKs responsible for the progression of specific cancers (Roskoski, Jr. 2004).

Receptor TKs & Cancer

Tyr Kinase	Cancer associations
EGFR family	
EGFR (HER-1)	Breast, ovary, lung, glioblastoma multiforme, and others
ERBB2 (HER-2)	Breast, ovary, stomach, lung, colon, and others
ERBB3 (HER-3)	Breast
ERBB4 (HER-4)	Breast, granulosa cell tumours
Insulin R family	
IGF-1R	Cervix, kidney (clear cell), sarcomas, and others
IRR, INSR	-
PDGFR family	
PDGFR-α	Glioma, glioblastoma, ovary
PDGFR-β	Chronic myelomonocytic leukaemia (CMML), glioma
CSF-1R	CMML, malignant histiocytosis, glioma, endometrium
KIT/SCFR	GIST, AML, myelodysplasia,mastocytosis, seminoma, lung
FLK2/FLT3	Acute myeloid leukaemia (AML)
VEGFR family	
VEGFR1	Tumour angiogenesis
VEBFR2	Tumour angiogenesis
VEGFR3	Tumour angiogenesis, Kaposi sarcoma, haemangiosarcoma
FGFR family	
FGFR-1	AML, lymphoma, several solid tumours
FGFR-2	Stomach, breast, prostate
FGFR-3	Multiple myeloma
FGFR-4	-
KLG/CCK family (CCK4)	
NGFR family	
TRKA	Papillary thyroid cancer, neuroblastoma
TRKB	
TRKC	Congenital fibrosarcoma, acute myeloid leukaemia
HGFR family	
MET	Papillary thyroid, rhabdomyosarcoma, liver, kidney
RON	Colon, liver
EPHR family	
EPHA2	Melanoma
EPHA1, 3, 4, 5, 6, 7, and 8	-
EPHB2	Stomach, oesophagus, colon
EPHB4	Breast
EPHB1, 3, 5, and 6	-
AXL family	
AXL	AML
MER, TYRO3	-
TIE family	
TIE	Stomach, capillary haemagioblastoma
TEK	Tumour angiogenesis
RYK family (RYK)	Ovarian cancer
DDR family (DDR1 and DDR2)	Breast, ovarian cancer
RET family (RET)	Thyroid (papillary and medullary), multiple endocrine neoplasia
ROS family (ROS)	Glioblastoma, astrocytoma
LTK family	
ALK	non-Hodgkin lymphoma
LTK	-
ROR family (ROR1 and ROR2)	-
MUSK family (MUSK)	-
LMR family (AATYK, AATYK 2, and 3)	-
RTK106	

Non-receptor TKs & Cancer

Tyr Kinase	Cancer associations
ABL family	
ABL1	Chronic myeloid leukaemia (CML), AML, ALL, CMML
ARG	AML
FRK family	
BRK	Breast
FRK	
SRMS	
JAK family	
JAK1	Leukaemias
JAK2	AML, ALL, T-cell childhood ALL, atypical CML
JAK3	Leukaemia, B-cell malignancies
JAK4	-
SRC-A family	
FGR	AML, CLL, EBV-associated lymphoma
FYN	-
SRC	colon, breast, pancreas, neuroblastoma
YES1	colon, melanoma
SRC-B family	
BLK	
HCK	-
LCK	T-cell ALL, CLL
LYN	
SYK family	
SYK	Breast
ZAP70	
FAK family	
FAK	adhesion, invasion and metastasis of several tumours
PYK2	adhesion, invasion and metastasis of several tumours
ACK family	
ACK1	-
TNK1	
CSK family	
CSK	-
MATK	
FES family	
FER	-
FES	
TEC family	
BMX	-
BTK	
ITK	-
TEC	-
TXK	

Table 1.1: Tyrosine kinases and their involvement in cancer (from: (Madhusudan & Ganesan 2004)). Since their discovery, many protein tyrosine kinases (PTKs) have been implicated in oncogenic transformation. This table exemplifies type of cancers where aberrations in PTKs have been reported.

1.2 The ErbB Receptor Family and Cellular Signalling

The ErbB family of RTKs is composed of EGFR (ErbB1/HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). Each receptor shares common structural features: an extracellular ligand binding domain, a hydrophobic transmembrane domain, and intracellular domain which contains tyrosine kinase activity and a C-terminal tail. Like other RTKs, ErbB receptors are activated through ligand-dependent induction of receptor dimerization. Ligand binding induces formation of both homo- and hetero-dimers of ErbB receptors, which then induces stimulation of the intrinsic tyrosine kinase activity of the receptors and triggers auto-phosphorylation and cross-phosphorylation of specific tyrosine residues in the C-terminal tail (Weiss & Schlessinger 1998). This creates docking sites for adaptor proteins and enzymes that initiate multiple signal transduction pathways (Yarden & Sliwkowski 2001). This intricate mode of action underlies the enormous potential for signalling diversification through the ErbB family. Moreover, each family member can trigger the activation of distinct, but overlapping, downstream signalling pathways and elicit equally varied cellular responses due to the multiplicity of ErbB ligands, the formation of various receptor dimers between different family members, and the recruitment of different subsets of intracellular signalling molecules (Hackel *et al.* 1999). Indeed, the proliferative potential of ErbB receptor signalling through different dimers has been studied in ErbB-free cells transfected with vectors for each ErbB member alone or in combination (Pinkas-Kramarski *et al.* 1996,Arteaga 2001). In this system, the most potent proliferative signal is elicited by ErbB2/ErbB3 dimers in response to HRG (Figure 1.1). Thus, the ability of ligand-activated ErbB receptors to homo-dimerize or dimerize with other family members provides several possible combinatorial interactions that can modulate the potency of signal transduction. Nonetheless, there is still much to be learnt about signal diversification by the ErbB family and the specific responses to their particular ligands.

Several growth factors bind with varied affinities and specificities to different ErbB family members. These ErbB-specific ligands can be divided into three groups. The first group consists of EGF, transforming growth factor-α (TGF-α) and amphiregulin (AREG), which bind specifically to EGFR. Heparin-binding EGF-like growth factor (HBEGF), betacellulin and epiregulin can bind both EGFR and ErbB4. The third group, composed of neuregulins such as heregulin (HRG) and neu

differentiation factor (NDF), are specific ligands for ErbB3 and ErbB4 (Alroy & Yarden 1997,Olayioye *et al.* 2000). Although ErbB2 remains an orphan receptor with no specific ligand described to date, it is the preferred dimerization partner for the other ErbB family members, possibly acting as a common subunit of ErbB receptors rather than as a growth factor receptor itself (Karunagaran *et al.* 1996a,Graus-Porta *et al.* 1997) and playing a role as a potentiator of ErbB receptor signalling (Beerli *et al.* 1995,Graus-Porta *et al.* 1995). Furthermore, ErbB3, like ErbB2, only becomes activated through the formation of hetero-dimers with other members of the family, as it lacks intrinsic kinase activity due to substitutions of critical residues in the receptor's kinase domain (Guy *et al.* 1994). These observations highlight the importance of receptor hetero-dimerization in the ErbB family. Nevertheless, overexpression of a specific receptor can bias dimer formation, such as in the case of ErbB2, which can spontaneously homodimerize, induce ligand-independent signalling and induce the transformation of NIH 3T3 fibroblasts when overexpressed (Di Marco *et al.* 1990).

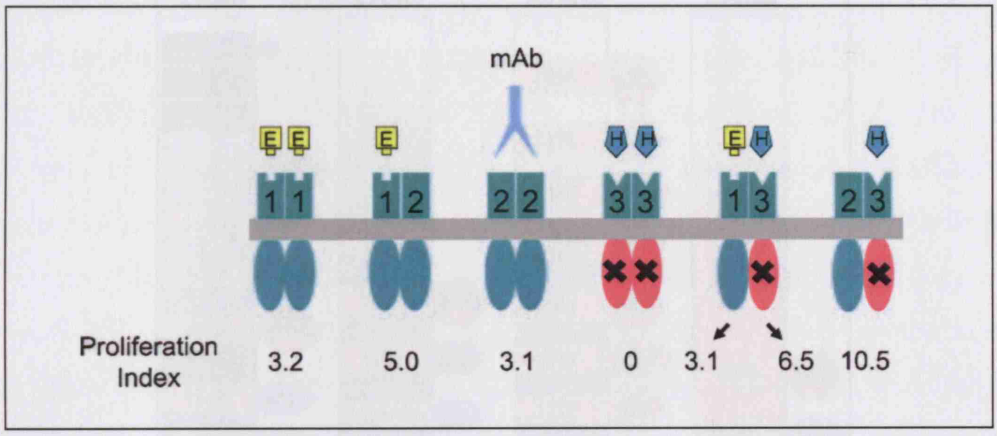

Figure 1.1: Proliferative potential of ErbB receptor dimers (from: (Arteaga 2001). E (EGF) or H (HRG) binding induce formation of different homo- or hetero-dimers. Each receptor combination yields different signalling potencies, as represented by their proliferative index shown below. The proliferative index was measured by transfecting ErbB receptor family members alone or in combination into an ErbB-null, interleukin-3 (IL-3)-dependent myeloid cell line. It represents the signal obtained in growth assays (MTT assay) after 48 hour incubation with EGF or HRG, and was calculated relative to the effect of IL3 (Pinkas-Kramarski *et al.* 1996). The impaired kinase activity of ErbB3 is illustrated by 'x', and mAb is a monoclonal antibody used in the study to induce the formation of ErbB2 homo-dimers.

As previously mentioned, each ErbB receptor has multiple sites for tyrosine phosphorylation, each acting as a binding site for specific intracellular proteins that in turn initiate distinct signal transduction cascades (Figure 1.2). ErbB binding proteins include adaptor proteins such as Shc, Crk, Grb2, Grb7 and Gab1; kinases such as phosphatidylinositol 3-kinase (PI3K) and Src and Chk; and the phosphatases Shp1 and Shp2 (Olayioye *et al.* 2000). Whereas all ErbB receptors have binding sites for Shc, other effector proteins such as Eps15 and Cbl only bind EGFR, and these are thought to be involved in receptor downregulation and termination of signalling (Levkowitz *et al.* 1996,Confalonieri *et al.* 2000). In addition, while EGFR and ErbB2 lack binding motifs for PI3K, ErbB3 has multiple binding sites for the PI3K regulatory subunit p85, and is therefore thought to be the most potent activator of the downstream PI3K signalling pathway (Hellyer *et al.* 1998).

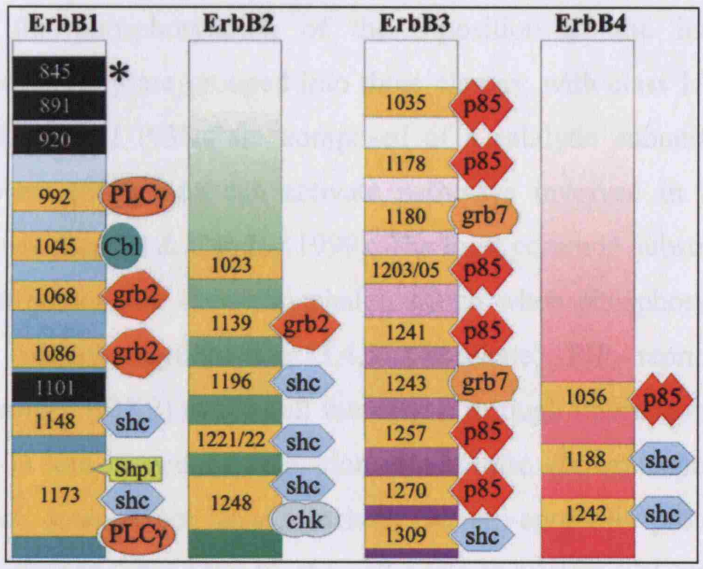

Figure 1.2: Phosphotyrosine binding sites and adaptor proteins recruited to the ErbB receptor family (from: (Olayioye *et al.* 2000)). Tyrosine auto-phosphorylation sites are shown in yellow, and sites for the Src kinase are shown in black.

Two of the major signalling cascades activated downstream of ErbB receptors are those involving the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K), which together regulate cell proliferation, apoptosis and survival (Olayioye *et al.* 2000,Yarden & Sliwkowski 2001). Activation of the MAPK pathway is typically achieved through the ligand-induced recruitment of the Grb2/Sos complex, which can link ErbB receptors to Ras by stimulating the exchange of GDP for GTP. Activated Ras binds to and promotes Raf activation, which then phosphorylates MEK1 and MEK2 dual specificity kinases. MEKs in turn phosphorylate the MAP kinases ERK1 and ERK2. MAPK then translocates into the nucleus where it can phosphorylate and activate transcription factors (Hunter 2000). Alternatively, activated Ras can bind PI3K to activate this signalling pathway (Rodriguez-Viciana *et al.* 1996).

PI3K pathway is also activated downstream of ErbB receptors by binding to phosphotyrosine residues in the ErbB3 receptor, resulting in its activation and the generation of phosphatidylinositol-3-phosphates (PI3Ps) which can activate downstream kinases such as PDK1 and Akt. PI3Ks are a subfamily of lipid kinases that catalyze the phosphorylation of the 3-position of the inositol ring of phosphoinositides. They are grouped into three classes, with class I being the most widely studied. Class I PI3Ks are composed of a catalytic subunit (p110) and a regulatory subunit (p85), and can activate pathways involved in a multitude of cellular responses (Rameh & Cantley 1999). The most common substrate for PI3K is PIP_2 (phosphatidylinositide -4,5- phosphate), which when phosphorylated by PI3K becomes PIP_3 (phosphatidylinositide -3,4,5- phosphate). PIP_3 recruits the kinases PDK1 (and possibly PDK2) to the cell membrane through interaction with their PH domain. PDKs in turn induce the activation of Akt (also known as protein kinase B, or PKB), whose downstream targets include the pro-apoptotic proteins BAD and caspase 9, the p53 regulator MDM2, the cell cycle regulators p21 and p27, and the p70/S6 kinase regulator mTOR, among others (Vanhaesebroeck & Waterfield 1999,Vivanco & Sawyers 2002).

In addition to the traditional MAPK and PI3K signalling, ErbB receptors can propagate signals through other signalling cascades that are regulated by multiple loops and extensive cross-talk with non-ErbB receptors, adding an extra layer of complexity to the study of ErbB signalling. For example, ErbB can integrate signalling events originating from other receptor families, such as receptors involved

in cytokine, integrin, insulin and G-protein coupled signalling (Alaoui-Jamali & Qiang 2003). An additional complication in ErbB2 signalling comes from recent findings where ErbB2 expression was found in the nucleus of human breast cancer cell lines, where it can associate with specific sequences in the promoter of the *COX-2* gene and induce its expression (Wang *et al.* 2004). Whilst a number of other RTKs have been found in the nucleus, including all other ErbB family members, the nuclear function of these receptors is largely unknown. The above study addressed this question by developing a cloning strategy that involves chromatin immunoprecipitation to identify specific DNA sequences as nuclear targets for RTKs and will surely stimulate further studies in the area of ErbB signalling. A summary of signalling through ErbB receptors is represented in Figure 1.3.

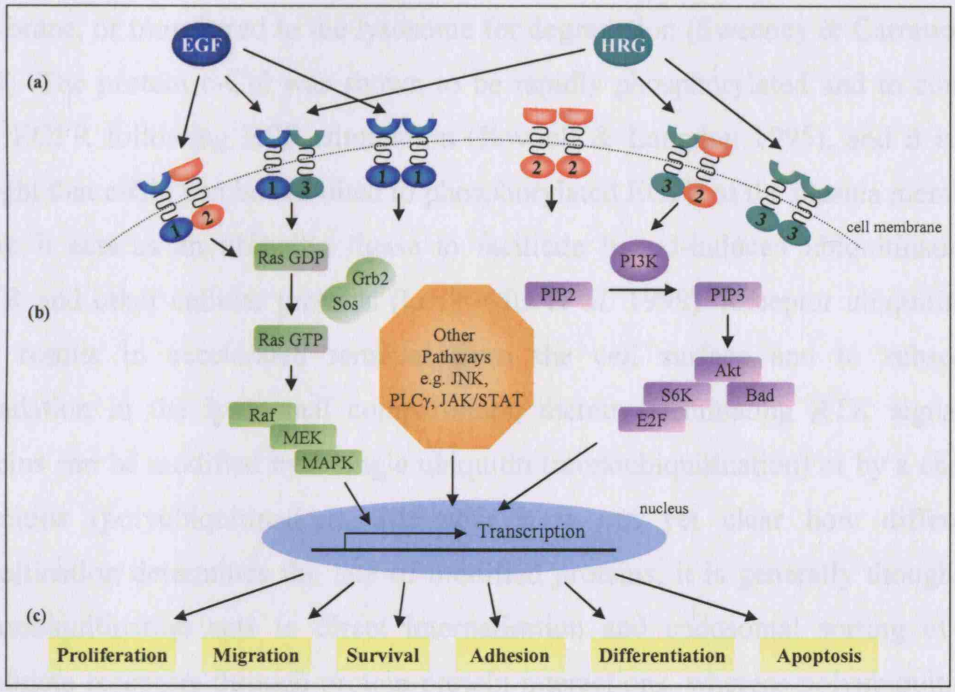

Figure 1.3: Intracellular signalling pathways downstream of ErbB receptors (adapted from: (Yarden & Sliwkowski 2001). The ErbB signalling network can be divided into three layers (Yarden & Sliwkowski 2001): **(a)** the input layer, comprised of ligands and their specific receptors. ErbB2 has no known ligands, and ErbB3 is catalytically inactive; **(b)** the signal-processing layer, comprised of receptor binding proteins and other intracellular proteins involved in signal propagation through a cascade of consecutive phosphorylation events leading to activation or repression of transcription; and **(c)** the output layer, which consists of specific cellular responses ranging from cell division and migration to adhesion, differentiation and apoptosis. Such responses are dependent on cellular context as well as the specific ligand and ErbB dimer that initiated the signal transduction pathway.

Finally, as well as positively activating pathways, such as the MAPK and PI3K described above, receptor activation also initiates mechanisms that will ultimately terminate signalling. In addition to protein de-phosphorylation by specific phosphatases, the main negative regulatory mechanisms for RTKs involves ligand-induced receptor endocytosis, which is coupled to degradation of both the receptor and the cognate growth factor (Waterman & Yarden 2001). RTKs are removed from the cell surface via clathrin-dependent endocytosis and degraded in the lysosomal compartment, while many intracellular proteins are targeted for degradation in the proteasome. Many of these regulatory steps are controlled by ubiquitination, a protein modification whereby the protein ubiquitin is covalently attached to a lysine residue of a target protein. For instance, upon ligand binding, EGFR becomes localized to plasma membrane clathrin-coated pits, which then become internalized and delivered to endosomes. From there, EGFR can either be recycled to the plasma membrane, or transferred to the lysosome for degradation (Sweeney & Carraway, III 2004). The protein c-Cbl was shown to be rapidly phosphorylated and to complex with EGFR following EGF stimulation (Bowtell & Langdon 1995), and it is now thought that c-Cbl can be recruited to phosphorylated EGFR at the plasma membrane, where it acts as an ubiquitin ligase to facilitate ligand-induced ubiquitination of EGFR and other cellular proteins (Levkowitz *et al.* 1998). Receptor ubiquitination then results in accelerated removal from the cell surface and to subsequent degradation in the lysosomal compartment, thereby terminating RTK signalling. Proteins can be modified by a single ubiquitin (monoubiquitination) or by a chain of ubiquitins (polyubiquitination). Although it is not yet clear how differential ubiquitination determines the fate of modified proteins, it is generally thought that monoubiquitination acts to direct internalisation and endosomal sorting of cell-membrane receptors through protein-protein interactions, whereas polyubiquitinated proteins are recognized by the proteasome that mediates protein unfolding and degradation. It is currently thought that a single ubiquitin in RTKs carries internalization and degradation signals by binding to ubiquitin-interacting motifs (UIMs), found in many endocytic proteins such as Eps15, epsin and Hrs (Dikic & Giordano 2003). Indeed, the capacity of c-Cbl to promote receptor internalization is believed to depend on its ability to connect EGFR to Eps15, a mediator of clathrin-coated pit formation, which itself also becomes phosphorylated and is recruited to EGFR following EGFR activation (de Melker *et al.* 2004). It is thought that Eps15

may play a critical role in the recruitment of active EGFR into coated pit regions before endocytosis by serving as a docking molecule responsible for the recruitment of the clathrin adaptor protein complex AP-2 to the plasma membrane (van Delft *et al.* 1997). The degradation pathways discussed above have been described only for EGFR, but there is evidence that a newly described ubiquitin ligase, Nrdp1, may catalyse the degradation of ErbB3 and ErbB4 via the proteasome (Diamonti *et al.* 2002,Qiu & Goldberg 2002).

1.2.1 ErbB receptors and cancer

ErbB receptors normally play a key role in the development of the cardiovascular system, nervous system and mammary gland, as shown from mouse knockout and gene expression studies (Casalini *et al.* 2004). However, they have also been implicated in malignant transformation. EGFR has been shown to be overexpressed in bladder, breast, head and neck, kidney, non-small cell lung (NSCL), ovary and prostate cancers; ErbB2 is overexpressed in breast, cervix, colon, endometrial, oesophageal, lung, ovary, stomach and pancreatic cancers; ErbB3 is overexpressed in breast, colon, prostate and stomach cancers; and ErbB4 is overexpressed in breast cancer and granulose cell tumours of the ovary (Blume-Jensen & Hunter 2001,Roskoski, Jr. 2004,Madhusudan & Ganesan 2004). Furthermore, a number of EGFR mutants, such as EGFRvIII, which is constitutively active and shows impaired downregulation, have been identified in human tumours (Jorissen *et al.* 2003).

Because of their altered expression in tumours, targeting ErbB receptor activity is a growing area in the drug development program of many companies. The two main strategies which are at the most advanced development stage for targeting EGFR (and indeed other ErbB receptors) involve the use of specific monoclonal antibodies (mAbs) or small molecule tyrosine kinase inhibitors, and a number of such compounds are currently being tested. MAbs are generally directed to the external domain of the receptor to block ligand binding and receptor activation and/or induce receptor internalization, while TK inhibitors prevent the phosphorylation of the receptor's intracellular tyrosine kinase domain. Examples of EGFR-specific mAbs include Erbitux, ABX-EGF and hR3, and TK inhibitors targeting EGFR include Tarceva, CI-1003 and Gefitinib (Thomas & Grandis 2004). Gefitinib (or

Iressa, AstraZeneca) was found to inhibit the growth of some cancer-derived cells and tumour xenographs, but in clinical trials positive responses were only seen in 9 to 19% of NSCL cancer patients (El Rayes & LoRusso 2004). This variability in response was later found to be in part due to somatic mutations in the PTK domain of the EGFR gene, which rendered some patients more responsive to Gefitinib (Paez *et al.* 2004,Lynch *et al.* 2004). These findings suggest that patient screening may help to predict responsiveness to treatment and to obtain effective "individually tailored" cancer therapies.

Although aberrant expression of all ErbB family members has been implicated in breast cancer (Blume-Jensen & Hunter 2001), perhaps the most important family member in the development of oncogenic transformation of the breast is ErbB2. ErbB2 is overexpressed due to gene amplification in 25-30% of all breast cancers and this overexpression positively correlates with disease progression and stage, reduced survival, poorer response to therapy and increased metastasis (Slamon *et al.* 1987,Ross & Fletcher 1998,Ross *et al.* 2003). ErbB2 overexpression has been associated with tamoxifen-resistance in patients with oestrogen receptor (ER) positive breast tumours (Kurokawa *et al.* 2000,Dowsett *et al.* 2001,Shou *et al.* 2004). Thus, measuring ErbB2 is important for selecting optimal therapy and predicting prognosis in breast cancer patients. Current methods for evaluating ErbB2 levels include measuring protein overexpression by immunohistochemistry (IHC), measuring gene copy number by fluorescent *in situ* hybridization (FISH), and measuring serum antigen levels by enzyme-linked immunosorbent assay (ELISA). Of these, the FISH technique on frozen sections is thought to produce the most consistent results (Ross & Fletcher 1998), although there have also been reports suggesting that real-time PCR may also be suitable for measuring ErbB2 mRNA levels (Bieche *et al.* 2003).

The role of ErbB2 in oncogenic transformation is likely to be a result of its high efficiency as a signal transducer, thereby inducing increased mitogenic signalling through a number of mechanisms. For instance, ErbB2 overexpression has been shown to enhance EGF and HRG binding affinity through reduced ligand dissociation rates and to potentiate signalling of other ErbB members by delaying receptor endocytosis and increasing the recycling rate of receptor to the plasma membrane (Graus-Porta *et al.* 1995,Tzahar *et al.* 1996a,Tzahar *et al.* 1996b,Karunagaran *et al.* 1996b,Worthylake *et al.* 1999). In addition, when

overexpressed, ErbB2 can undergo spontaneous homo-dimerization and ligand independent signalling (Worthylake *et al.* 1999,Harari & Yarden 2000). These events allow prolonged receptor activation and signal duration, resulting in increased activation of the downstream pathways involved in proliferation, migration and survival, thereby contributing to malignant transformation. It is thought that ErbB2 can also affect the components of the cell cycle regulatory machinery, thereby inducing cellular transformation. In this context, ErbB2 mitogenic signalling has been associated with increased expression of cyclin D1, an essential factor of G1/S phase transition of the cell cycle. Upregulation of cyclin D1has been observed in cell lines that overexpress ErbB2 (Lee *et al.* 2000,Timms *et al.* 2002), and cyclin D1 was shown to be required for ErbB2-induced transformation, as demonstrated by the failure of the MMTV-ErbB2 transgene mice to induce mammary carcinomas in cyclin D1 knockout mice (Yu *et al.* 2001). ErbB2 overexpression has also been shown to potentiate cyclin E-cdk2 activity, possibly through degradation of the cell cycle inhibitor $p27^{Kip1}$ (Lane *et al.* 2000,Timms *et al.* 2002).

Based on the discovery of the significance of aberrant ErbB2 expression in breast cancer, the humanized anti-ErbB2 monoclonal antibody Herceptin (Genetech Corporation) was developed. This antibody has an excellent clinical anti-tumour profile and except for an observed risk of cardiac dysfunction (mainly in patients who have had previous exposure to the chemotherapy drug anthracycline or who show cardiac risk factors such as family history), Herceptin-treated patients only show mild side effects (Leyland-Jones 2002). When used in combination with chemotherapy, Herceptin increased overall response rate and response duration, and improved the median survival time by approximately 25% compared with chemotherapy alone (Baselga 2001,Slamon *et al.* 2001). In addition, pre-clinical experiments have shown that ErbB2 inactivation may enhance the benefit of tamoxifen action against ErbB2-overexpressing, tamoxifen-resistant breast cancer cells (Kurokawa *et al.* 2000). These and other favourable results from clinical trials have led to the approval of Herceptin by the US Food and Drug Administration (FDA) in 1998 for the treatment of ErbB2-positive breast cancers. Although the exact mechanism of action of Herceptin remains unclear, there have been suggestions that its anti-tumour effects are due to antibody-dependent cellular toxicity and ErbB2 receptor down-regulation as a result of increased endocytic degradation (Leyland-Jones 2002). While Herceptin is not thought to prevent

dimerization of ErbB to other ErbB receptors (Badache & Hynes 2004), this antibody has indeed been shown to decrease signalling downstream of ErbB receptors, as exemplified by findings that it induces inhibition of PI3K/Akt (Yakes *et al.* 2002) and up-regulation of the Cdk inhibitor $p27^{Kip1}$ (Sliwkowski *et al.* 1999).

However, as in the previously mentioned case of Gefitinib, not all ErbB2-positive patients respond to Herceptin treatment, with less than 35% of ErbB2-overexpressing breast cancer patients shown to respond to Herceptin as a single agent (Vogel *et al.* 2002). It has been suggested that loss of PTEN, a PIP3 phosphatase, in ErbB2 overexpressing tumours can be responsible for Herceptin resistance, and therefore the use of combination therapy of Herceptin and PI3K inhibitors may overcome PTEN-loss mediated resistance and yield better therapeutic response (Nagata *et al.* 2004). Indeed, it appears that combination therapy results in response rates higher than that seen with Herceptin alone. A phase II trial of herceptin and cisplatin in patients with previously treated metastatic breast cancer produced an overall response rate of 24%, which is higher than previously reported with either agent alone (Pegram *et al.* 1998). A large randomized study comparing Herceptin plus chemotherapy (paclitaxel or an anthracycline plus cyclophosphamide) with chemotherapy alone demonstrated an improved time to disease progression, rate and duration of response and survival for patients who received chemotherapy plus Herceptin (Slamon *et al.* 2001). Combination therapy targeting other ErbB members has also shown promising results. Simultaneous inhibition of EGFR and ErbB2 showed a more potent anti-proliferative effect on cancer cell lines (Motoyama *et al.* 2002), and Herceptin treatment in combination with the EGFR-specific TK inhibitor Gefitinib elicits improved blockade of proliferation, survival and signal transduction in breast cancer cells (Normanno *et al.* 2002). In this regard, it is unlikely that a single active RTK is exclusively responsible for malignant transformation. Thus, in order to improve the efficacy of ErbB2-dependent breast cancer therapy, it is necessary to fully understand the mechanisms of ErbB2-dependent transformation by characterizing in detail the mechanisms of ErbB2 signalling, the collaboration with other ErbB family members, the downstream signalling effectors, induced/repressed gene expressions and their interactions with other non-ErbB signalling pathways to identify new potential therapeutic targets.

1.3 ErbB2-Overexpressing Cell System

The use of cancer models is essential for the study of the mechanisms of malignant transformation and the properties of human cancer. Although the use of clinical biopsies obtained from patients may be more representative of the real state of illness, obtaining such samples is a highly invasive procedure and is undesirable for research purposes. In addition, such samples are very heterogeneous and do not yield large quantities of material for laboratory use. The use of cell lines as a tool for studying cancer may not be immediately obvious owing to the fact that cells in culture lack the cellular or tissue environment in which they normally grow, they are prone to genetic instability and may undergo genotypic/phenotypic alterations due to long term culturing in simplified conditions. However, when studying the events associated with cancer development using cancer models, it is important that the model used can, to the best possible level, match the experimental questions being asked. Indeed, a large proportion of the current knowledge on human cancer is based on *in vivo* and *in vitro* studies performed in established cell lines.

A number of cell lines have been developed for the study of breast cancer. These include cell lines derived from pleural effusions of patients with metastatic disease, (e.g. MCF-7, SKBr-3, BT 474 and SUM cell lines), from primary tumours (e.g. HCC cell line series), from metastasis sites (e.g. MDA-MB361 and MDA-MB435, cell lines derived from brain metastasis), and from tumours of patients carrying germ-line mutations (e.g. HCC1937, a BRCA1 mutant cell line). In addition, variant breast cancer cell lines obtained by selection of cell sub-populations or through different culture conditions have been described, such as MCF-7/LCC1 and MCF-7/LCC9 cells, which are MCF-7 cells showing hormone-responsiveness and hormone-resistance, respectively (Lacroix & Leclercq 2004). A number of studies have looked at how representative breast cancer cell lines are when compared to the tumours from which they are derived. In general, it is believed that breast cancer cell lines reflect the features of cancer cells *in vivo* (Lacroix & Leclercq 2004). Moreover, positive correlation has been found in breast cancer cell lines and their corresponding tumour tissues for morphological features, presence of aneuploidy, oestrogen, progesterone and ErbB2 receptor expression and p53 expression (Wistuba *et al.* 1998).

Another approach to develop relevant models of breast cancer involves the manipulation of normal cells, for example through the alteration of several

oncogenes or tumour suppressor genes to render cells immortalized and/or transformed. Unlike the above examples, which represent more advanced stages of breast cancer, genetic manipulation of normal cells potentially allows the characterization of early events involved in cancer development and the events associated with the expression of specific markers of breast cancer, such as ErbB2. In this context, a cellular model of breast cancer was developed to investigate the role of ErbB2 overexpression in cellular transformation, and this model has been used in the present study. The HB4a luminal epithelial cell line was derived from reduction mammoplasty tissue from a healthy patient and subsequently immortalized using a temperature-sensitive mutant of the SV40 large T antigen (Stamps *et al.* 1994). These cells display a non-transformed phenotype and still retain luminal epithelial markers and cell behaviour. HB4a cells were subsequently transfected with full length human *c-erbB2* cDNA under the control of the mouse mammary tumour virus long terminal repeat (MMTVLTR). Clones were selected by FACS for ErbB2 expression and a number of cell lines were generated, expressing different levels of ErbB2 (Harris *et al.* 1999). The commonest form of breast cancer, infiltrating ductal carcinoma (IDC), arises from luminal epithelial cells of the mammary gland, and therefore this cell system is ideal for the study of the early events associated with malignant transformation of the breast and with ErbB2 overexpression. In addition, these cell lines should share an identical genetic background thereby providing an appropriate and relevant control sample. These cells do not exhibit amplification at the ErbB2 "amplicon" (17q12-q21), allowing the study of ErbB2-related signalling events independently of the effects of other co-amplified genes.

The ErbB2-overexpressing clone C3.6 (in conjunction with the parental cell line HB4a) was chosen for this study as it was shown to express levels of surface ErbB2 similar to those observed in human breast cancers (Harris *et al.* 1999). C3.6 cells do not induce the formation of tumours after inoculation into nude or SCID mice, suggesting that ErbB2 alone cannot fully transform cells. This supports the notion that additional genetic alteration events are required for the development of cancer, so it is unlikely that ErbB2 alone can induce transformation. However, the ErbB2-overexpressing cells were hyper-proliferative when compared to control (HB4a cells) and showed increased anchorage-independent growth, growth factor responsiveness and ligand-independent ErbB2 homo-dimerization and phosphorylation (Harris *et al.* 1999).

This system was further characterized by Timms *et al* with respect to the effect of ErbB2 overexpression on mitogenic signalling and cell cycle progression (Timms *et al.* 2002). This work showed that both EGF and HRG could activate MAPK and PI3K signalling, with higher activation of MAPK signalling in the ErbB2-overexpressing C3.6 cells. Furthermore, EGF and HRG induction of c-myc, cyclin D1, cdk6 and cyclin E were higher in C3.6 cells, whilst the cdk inhibitor $p27^{Kip1}$ was found to be downregulated in C3.6 cells and further downregulated by growth factor treatment. As previously discussed, similar changes have been shown in other models of ErbB2 overexpression (Lee *et al.* 2000,Lane *et al.* 2000,Yu *et al.* 2001). These combined changes support the notion that ErbB2 promotes a more rapid progression through the cell cycle. This cell system was also studied in the context of gene expression using microarrays in our laboratory (White *et al.* 2004) and by others (Mackay *et al.* 2003), and these studies will be discussed in section 1.4.6.

In conclusion, preliminary work indicates that this cell system is useful for the study of breast cancer and was used in this study to examine the effects of ErbB2 overexpression on gene expression and the responsiveness to ErbB-specific growth factors.

1.4 Overview of Microarray Technology

Of the estimated total number of genes encoded in each mammalian cell, only a portion is being actively transcribed at a given time. Such genes determine the structural and biological behaviour of a cell, and therefore understanding variations in these transcriptional events may help to resolve the mechanisms by which diseases develop. As a result of the Human Genome Project, there has been a massive increase in the amount of information available about the DNA sequences of human genes. However, the challenge now facing scientists is to identify the function of previously unknown sequences, to further characterize the known genes and to understand their expression patterns, function and regulation in normal and diseased states. Microarray technology is a valuable tool that allows the measurement of the relative expression levels of thousands of genes in a cell or tissue in a single experiment. Thus, the expression profile of a particular disease such as cancer can be determined and specific genes involved in that condition identified when compared

to the normal state. This may ultimately result in the understanding of the genetic events leading to malignancy, and in the identification of potential drug targets.

Traditional methods for gene expression measurements such as Northern blots can be time-consuming and labour-intensive and are not practical for application on a very large scale. Methods suitable to profile the expression of a large number of genes include serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS) and microarrays. Although both SAGE and MPSS allow direct quantification of the number of mRNA molecules expressed in a tissue or cell (Pollock 2002), the most widely used technology for high-throughput measurement of gene expression is microarray technology, owing to its relative simplicity when compared to the other techniques.

1.4.1 Oligonucleotide & cDNA: the two major array platforms

There are two main platforms for microarray analysis of gene expression: oligonucleotide arrays and cDNA arrays. Oligonucleotide arrays (trademarked as GeneChip® by Affymetrix Inc.) use small 25 base pair gene fragments as the probe to be spotted onto an array. These probes are constructed using a combination of photolithography, and solid-phase DNA synthesis techniques. In this process, a solid support is coated with a covalent linker molecule terminated with a photolabile protecting group. Light is directed through a mask to deprotect and activate selected sites, and the surface is then flooded with a solution containing adenine, thymine, cytosine or guanine. Coupling occurs only in those regions on the glass that have been deprotected through illumination. The coupled nucleotides also contain the light-sensitive protecting group thereby allowing this process to be repeated, activating different sets of sites and coupling different bases allowing arbitrary DNA probes to be constructed at each site. While probes are selected to have little cross-reactivity with other genes so that non-specific hybridization can be minimized, some non-specific hybridization may occur. In order to avoid this, a second probe that is identical to the first except for a mismatch at its centre is placed next to the first. This Perfect Match/Mismatch (PM/MM) probe strategy provides controls that allow the subtraction of both background and cross-hybridization signals. Typically, 11 to 16 of these probe pairs, each interrogating a different part of the sequence of a gene, are spotted onto the array, and the intensity information from the value of each

of the probes are combined together to obtain an average expression value (Lipshutz *et al.* 1999). Samples for hybridization onto oligonucleotide arrays consist of biotin-labelled cRNA, and hybridization signals are developed using immunochemistry and detected using a high-resolution scanner. It is currently possible to measure the expression of up to 40,000 human genes in a single hybridization experiment using this system. However, only one sample can be hybridized to each array, therefore the two (or more) samples being compared are labelled with the same detectable tag and individually hybridized to different arrays, requiring a large number of arrays per experiment and significantly increasing the cost. Another oligonucleotide-based microarray platform makes use of ink-jet technology to synthesize longer (60-70mer) oligonucleotides. The advantage of this platform over the 25-mer arrays is its higher hybridization specificity due to the longer length oligos as well as its flexibility, since no new masks are required to synthesize oligonucleotides when adding or changing the gene content on the arrays (Hughes *et al.* 2001).

cDNA arrays are typically produced by depositing PCR products, made from cDNA clones, on modified glass slides. These products usually represent sequences chosen directly from databases such as GenBank and UniGene, but ESTs or randomly chosen cDNA from a particular library of interest can also be used. In general, PCR products are several hundred to a few thousand base pairs, and one product (or sometimes a few different products) is used to probe each gene. These arrays can be produced by individual investigators or core facilities, but this requires more sophisticated and costly equipment, so these arrays are generally produced commercially. In a cDNA array experiment, total RNA or mRNA from a control (reference) and an experimental (test) sample are isolated and reverse transcribed using oligo-dT primers in the presence of the fluorescent dyes Cy3- (green) or Cy5- (red) tagged dCTP. Thus, the RNA from the test and reference samples are differentially labelled. Both samples are then pooled and allowed to competitively hybridise to cDNA clones (or probes) on the glass slide. Laser excitation of the hybridized targets yields emission with a characteristic spectra for each Cy dye, which can be scanned and imported into a software in which images are pseudo-coloured and merged (Duggan *et al.* 1999). Thus, the gene activity, as measured by relative mRNA abundance, is determined by the ratio of the fluorescence of the test sample over the fluorescence of the reference sample (Figure 1.4).

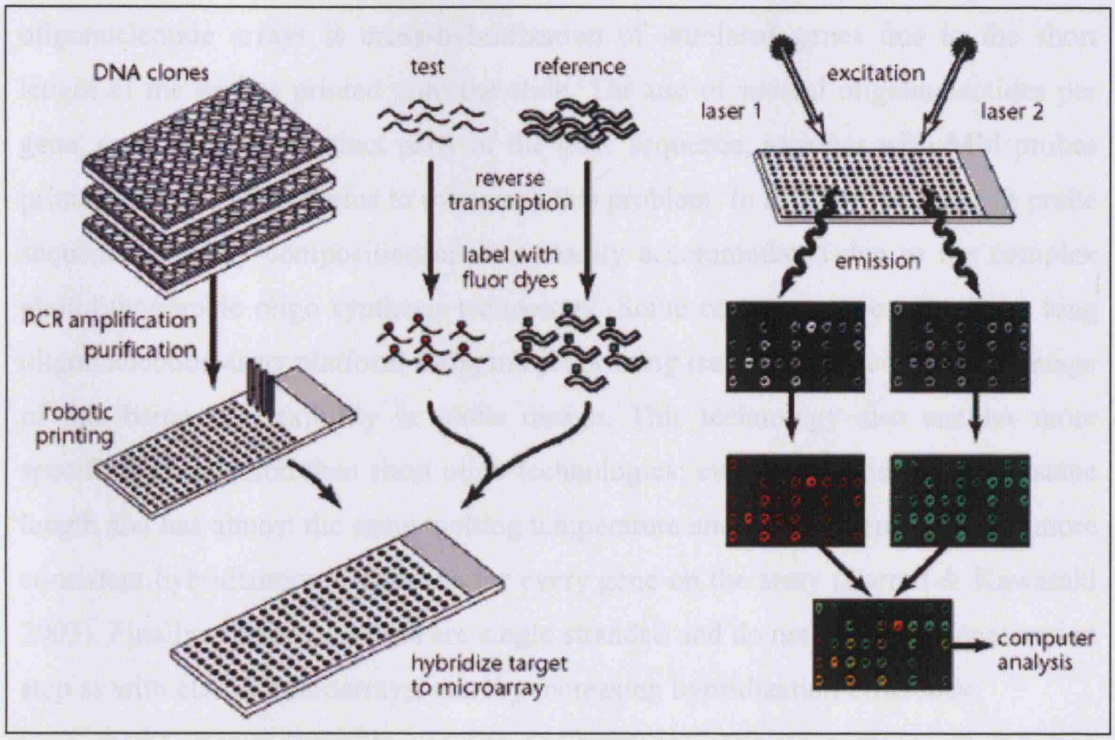

Figure 1.4: Schematic representation of a typical cDNA microarray experiment (from (Duggan et al. 1999). Templates for genes of interest are PCR-amplified and printed onto a coated glass slide using a computer controlled robot. RNA is isolated from a test and a reference sample and fluorescently labelled with either Cy3- or Cy5-dCTP during the reverse transcription step. Differentially labelled cDNA from both samples are then pooled together and allowed to competitively hybridize to the clones on the array. Slides are then washed and subject to laser excitation, yielding an emission with distinct spectra for each dye which is then measured using a scanning confocal laser microscope. Images are pseudo-coloured and merged, and fluorescent intensity is measured for each channel. This data is then viewed as a ratio (Cy3/Cy5) which indicates the level of expression of the test sample relative to the reference.

1.4.2 Comparison between oligonucleotide and cDNA microarrays

Both cDNA and oligonucleotide microarray platforms offer both advantages and disadvantages with respect to many factors such as the starting amount of probe required, cost, time, data acquisition and analysis. One of the main drawbacks of oligonucleotide arrays is cross-hybridization of unrelated genes due to the short length of the probes printed onto the slide. The use of several oligonucleotides per gene, each querying distinct parts of the gene sequence, together with MM probes printed onto the array seems to overcome this problem. In addition, changes in probe sequence or array composition are not readily accommodated due to the complex photolithographic oligo synthesis technology. Some companies are adopting a long oligonucleotide array platform using ink-jet printing technology, the major advantage of this being its flexibility in probe design. This technology also enables more specific hybridization than short oligo technologies: every nucleotide is of the same length and has almost the same melting temperature and G-C content, enabling more consistent hybridization conditions for every gene on the array (Barrett & Kawasaki 2003). Finally, oligonucleotides are single stranded and do not require a denaturation step as with cDNA microarrays, thereby increasing hybridization efficiency.

cDNA arrays typically use the test/reference ratio as a means to measure relative gene expression, and so different experiments are likely to have different "reference" samples therefore making cross-comparison of different data sets (Shi *et al.* 2004) difficult. However, cDNA arrays offer the advantages of being relatively easy to "custom make" at considerably lower costs. Also, it is not necessary to have sequence information before setting up the arrays, as PCR products can be synthesized using universal primers and interesting genes can be sequenced after array analysis. The large size of PCR products is also helpful in enabling stringent hybridization conditions and lowering cross-hybridization of unrelated genes.

1.4.3 The various stages of microarray experiments

There is a great deal of apprehension among biologists regarding microarray technology. Whereas the use of microarrays to generate new insights into biological systems is very exciting, most users become frustrated by the data analysis aspect, which is often too complex to understand or to perform faithfully (Leung *et al.* 2001). Collaborations with experts in other fields may be helpful, but frequently data mining

experts and biologists do not work with the same mindset and this may hinder the discovery of new, biologically significant data. Thus, collaborations can be helpful, but they cannot replace a good foundation in data analysis.

To obtain maximum results from microarray experiments, every step of the process must be carefully thought out (Figure 1.5). The way the experiment is designed, performed and analysed must be the best possible in order to directly address the questions being asked, ensuring that the interpretation of results is accurate and reliable. Thus, biologists must carry out microarray experiments in the most efficient way, and like any high-throughput technology, must ensure sufficient quality control for each step in the study. In this section, some of the main steps to be considered in a microarray experiment will be briefly discussed in turn. Since spotted cDNA arrays were used in this study, this section will focus on two-colour arrays rather than oligonucleotide arrays.

1.4.3.1 Experimental design

The main focus on microarray data analysis has been on the late stage data mining. However, the earlier stages should not be neglected, as experimental design can affect the quality of final stage analysis. For example, badly designed experiments may leave the investigator unable to answer the question of interest with the data that has been collected, or even introduce a bias in the data that may compromise the interpretation of the results (Yang & Speed 2002). Important considerations before starting an experiment include how samples will be prepared, the aim of the experiment (i.e. what are the questions trying to be answered and how will this experiment answer them), the amount of sample and the number of slides available, the number of replicates, and the cost.

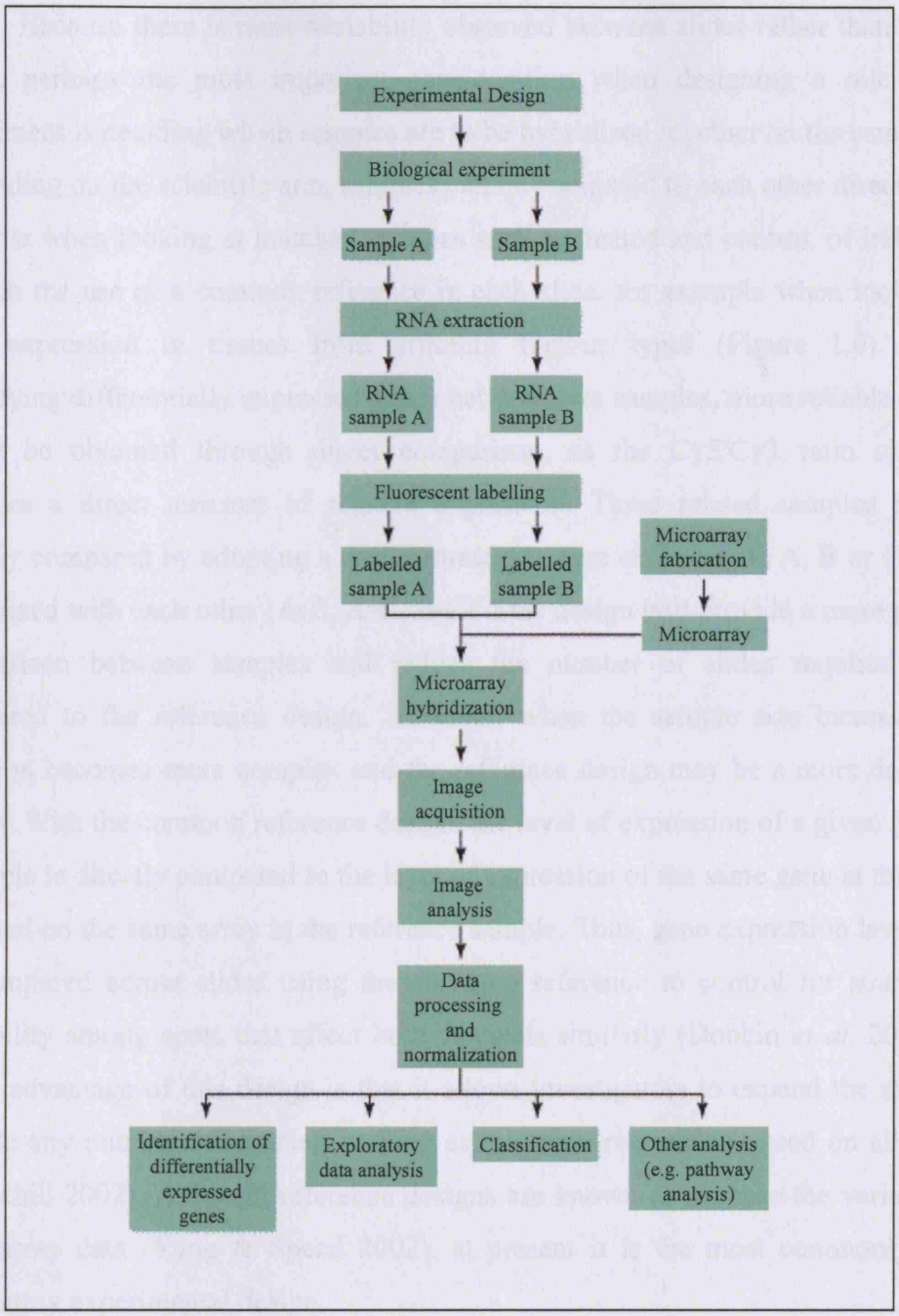

Figure 1.5: Typical flow of a microarray experiment (from: (Leung & Cavalieri 2003)). Before starting a microarray experiment, it is critical to design the experiment in the best possible way to obtain the correct answers for the experimental question being asked. Following sample preparation, labelling hybridization and image acquisition, image analysis is carried out to obtain raw signals for every spot. Poor quality data are filtered out and the remaining high quality data is normalized. Finally, data can be analysed using various statistical techniques to identify differential gene expression, perform various exploratory analyses, classify samples according to their disease subtype or carry out pathway analysis.

Because there is more variability observed between slides rather than within slides, perhaps the most important consideration when designing a microarray experiment is deciding which samples are to be hybridized together on the same slide. Depending on the scientific aim, samples can be compared to each other directly, for example when looking at matched samples such as treated and control, or indirectly through the use of a common reference in each slide, for example when looking at gene expression in tissues from different tumour types (Figure 1.6). When identifying differentially expressed genes between two samples, more reliable results would be obtained through direct comparison, as the Cy5/Cy3 ratio obtained provides a direct measure of relative expression. Three related samples can be directly compared by adopting a design strategy where each sample A, B or C is co-hybridized with each other (A-B, A-C, B-C). This design will provide a more precise comparison between samples and reduce the number of slides required when compared to the reference design. However, when the sample size increases the situation becomes more complex and the reference design may be a more desirable option. With the common reference design, the level of expression of a given gene in a sample is directly compared to the level of expression of the same gene at the same spot and on the same array in the reference sample. Thus, gene expression levels can be compared across slides using the common reference to control for sources of variability among spots that affect both channels similarly (Dobbin *et al.* 2003). A major advantage of this design is that it allows investigators to expand the study to include any number of samples, as long as the same reference is used on all slides (Churchill 2002). Although reference designs are known to increase the variance of microarray data (Yang & Speed 2002), at present it is the most commonly used microarray experimental design.

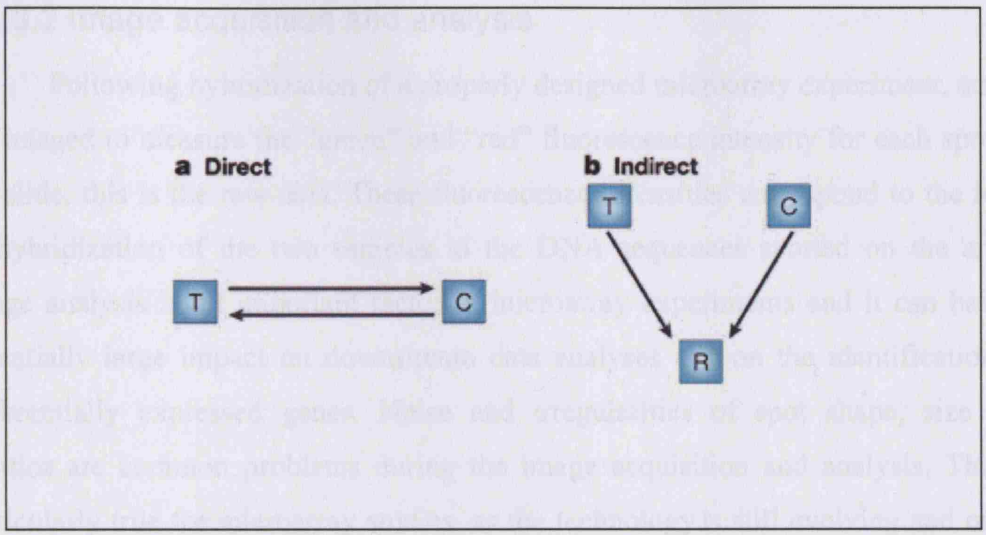

Figure 1.6: Direct vs. indirect microarray experimental design (from: (Yang & Speed 2002). In the direct comparison design **(a)**, the two samples T and C are co-hybridized on the same slide and therefore can be compared directly to each other. In the indirect comparison **(b)**, samples T and C are co-hybridized with a common reference sample (R) and therefore expression levels are measured separately on different slides. The relative expression is determined by the ratio (T/R)/(C/R).

Another concern in microarray experiments is the differences in labelling efficiency between Cy3 and Cy5. Although background correction and normalization can adjust for dye-related differences that are not gene-specific, it is unlikely these normalization steps can be done equally well for every spot on every slide leaving no residual colour bias. To try and overcome this dye bias, a "dye-swap" experimental design has been proposed, where each hybridization is performed twice, with the dye assignments reversed in the second hybridization (Yang & Speed 2002). It has been suggested that the dye-swapping strategy can introduce significant variability in the experiment (Ahmed *et al.* 2004), but the use of a reference experimental design could eliminate the need for dye-swapping (Dobbin *et al.* 2003). However, this is still a matter of debate as other reports suggest that even using the reference design, dye bias can significantly influence measured changes in gene expression (Dombkowski *et al.* 2004). Therefore, the decision of whether to use dye-swap or reference design (or both) should be based on the analysis of the noise introduced by each method in a pilot experiment. In any case, the highest number possible of technical and biological replicates should always be used.

1.4.3.2 Image acquisition and analysis

Following hybridization of a properly designed microarray experiment, arrays are imaged to measure the "green" and "red" fluorescence intensity for each spot on the slide, this is the raw data. These fluorescence intensities correspond to the level of hybridization of the two samples to the DNA sequences spotted on the array. Image analysis is an important factor in microarray experiments and it can have a potentially large impact on downstream data analyses and on the identification of differentially expressed genes. Noise and irregularities of spot shape, size and position are common problems during the image acquisition and analysis. This is particularly true for microarray studies, as the technology is still evolving and many researchers are using home-made arrays. Therefore, users need to be able to acquire quality data and to control for imperfections that happen during printing and hybridization.

The three fundamental processes of image analysis are gridding, segmentation and information extraction (Yang *et al.* 2001). Gridding is the process by which each spot is located on the slide, and the fully automated state of this part of the procedure permits high-throughput analysis. This step also distinguishes the "real" spots from artefacts such as precipitated probe or contaminants such as dust on the surface of the slide, and allows for these artefacts to be flagged out of data analysis. Segmentation is the process that allows the classification of pixels within a spot-containing region either as foreground (true signal) or background. Current segmentation or quantitation methods for microarray images can be divided into four groups: (i) fixed circle segmentation, (ii), adaptive circle segmentation, (iii) adaptive shape segmentation, and (iv) histogram segmentation (Yang *et al.* 2000). It has been reported that the choice of segmentation method can yield significant differences in data precision and in the number of differentially expressed genes, and that the histogram method performed best for within- and between-slide variability (Ahmed *et al.* 2004). Information extraction involves calculating the foreground fluorescence intensity pairs (i.e. red and green) and the background intensities for each spot on the array. For microarrays, it is important that the background is calculated locally for each spot rather than globally for the entire image, as uneven background can often arise during the hybridization process. Different algorithms can be used to estimate the background signals due to the non-specific hybridization on the glass, and

therefore one can perform background correction prior to data analysis. Moreover, Yang *et al* (Yang *et al.* 2000) compared a number of existing background correction and segmentation methods and found that the choice of background correction method has a greater impact on the log-intensity ratios than the segmentation method. Thus, researchers have a number of image analysis options and must decide which option is the best to ensure high quality data for further analysis.

1.4.3.3 Normalization

The data extracted from image analysis must be pre-processed to exclude poor quality spots and normalized to remove as many systematic errors as possible before downstream analysis. Spots with intensity lower than the background should be excluded from the analysis, and the intensity ratios should be log-transformed so that up-regulated and down-regulated values are on the same scale. Normalization is necessary to adjust for differences in labelling and detection efficiencies for the fluorescent labels and for differences in the quantity of the starting RNA for the two samples examined in the experiment, allowing between-slide comparisons. These problems can cause a shift in the ratio of Cy5 to Cy3 and the intensities must be re-scaled before the experiment can be properly analysed (Leung & Cavalieri 2003).

There are three commonly used normalization strategies, all of which are based on underlying assumptions regarding the data, and therefore the strategy used for each experiment should be adjusted to reflect both the system under study and the experimental design (Quackenbush 2001). The first approach uses total measured fluorescent intensity, and it is based on the assumption that the total mass of RNA labelled with Cy3 or Cy5 is equal. While the intensity for any one spot may be higher in one channel than the other, when averaged over thousands of spots in the array, these fluctuations should average out (Quackenbush 2002). A second approach uses linear regression analysis, where many of the genes on the array would be expected to be expressed at nearly constant levels, and a scatter plot of Cy3 vs. Cy5 intensities should have a slope of one. Under this assumption, one can use regression analysis techniques to calculate the slope. This is then used to re-scale the data and adjust the slope to one. The third approach assumes that a set of essential genes, such as housekeeping genes, exist, and for these the transcription levels are the same (Chen 1997). The Cy5 to Cy3 ratio for these genes can then be modelled and the mean

expression ratio adjusted to one, and the calculated confidence limits can be used to identify differentially expressed genes (Quackenbush 2001). In many experiments, however, intensities are non-linear. It is a common observation that the $\log_2$(ratio) values have a systematic dependence on intensity, an event that can be seen in MA-plots (Quackenbush 2002,Yang *et al.* 2002). An MA-plot is a graphical way to visualize ratios and fluorescence intensity at the same time, where the intensity log-ratio M=$\log_2$Red/Green is plotted versus the mean log intensity A=$\log_2\sqrt{}$Red/Green (Dudoit *et al.* 2002). The dependence of $\log_2$ ratios on intensity is best illustrated in an experiment where identical mRNA samples are labelled with Cy3 and Cy5 and subsequently hybridized to the same slide (self-self comparison), as described by Dudoit *et al* (Dudoit *et al.* 2002). In a "perfect" self-self comparison, the intensity log ratios M in the MA-plot should be evenly distributed around zero across all intensity values A. However, this is rarely the case, and systematic error often manifests itself in terms of non-zero log ratios M. In this regard, the local regression technique LOWESS (LOcally WEighted Scatterplot Smoothing) is believed to be superior to other normalization methods (Leung & Cavalieri 2003), because it can remove intensity-dependent effects on the $\log_2$(ratio) values by detecting systematic deviations in the MA-plot and correcting them (Quackenbush 2002). Figure 1.7-a shows an MA-plot for a microarray data set showing clear intensity-dependent dye bias. The same dataset after LOWESS normalization is shown in Figure 1.7-b (Yang *et al.* 2002).

In all these normalization strategies, the variance of the normalized set can be used to generate estimates of the expected variance, leading to predicted confidence intervals. There are a number of other transformations that can be applied to expression data, including specific techniques that have been developed for data derived from particular platforms (Li & Wong 2001) and complex methods looking at the development of error models based on the analysis of repeated hybridizations (Rocke & Durbin 2001). Regardless of all the sophisticated strategies available to correct for systematic errors, no normalization technique can compensate for poor quality data. Thus, every effort must be made in each step of the process to minimize variation.

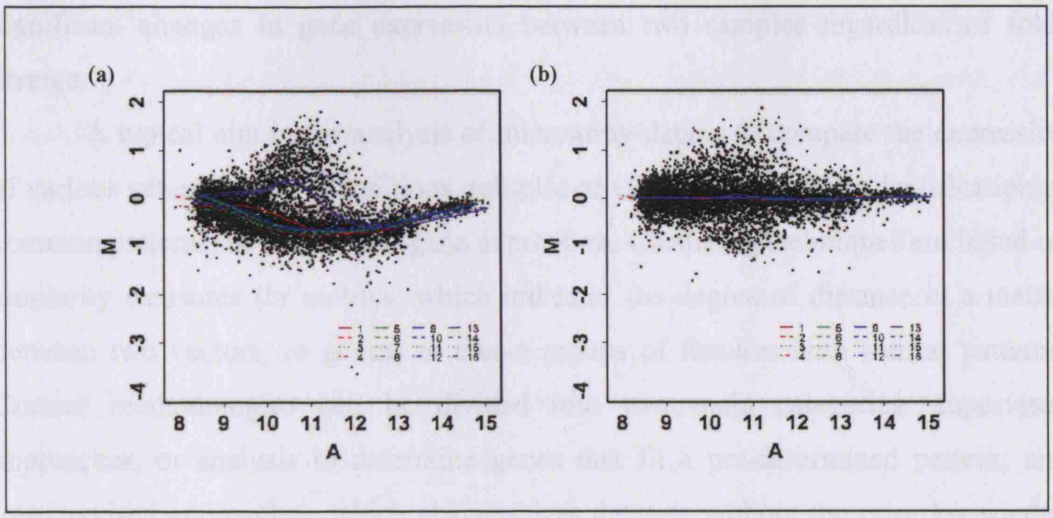

Figure 1.7: Example of an MA-plot before and after LOWESS normalization (from: (Yang *et al.* 2002). The clear curvature in the MA-plot in the left panel strongly suggests the existence of an intensity-dependent dye bias in this date set. The right panel shows the MA-plot for the same dataset following LOWESS normalization.

1.4.3.4 Data mining

Following normalization, data are typically analysed to identify genes that are differentially expressed. Many published studies have used a post-normalization cut-off of two-fold up- or down-regulation to define differential expression. This measure is not ideal for microarray studies as one cannot quantify the absolute level of transcripts, only the amount relative to the control used in a particular experiment. In addition, the fold-change cut-off method takes little consideration in variability between replicates and therefore the likelihood of false positive or false negative results is increased. A more efficient way to determine whether gene expression changes are experimentally significant was developed by Tusher *et al* (Tusher *et al.* 2001). The SAM (Significance Analysis of Microarrays) method identifies genes with statistically significant changes in expression by assigning a score to each gene on the basis of changes in gene expression relative to the standard deviation of repeated measurements. Genes with a score above a threshold are potentially significant genes. SAM then uses permutations of replicate measurements to calculate the percentage of significantly changing genes identified by chance (the false discovery rate, or FDR). Using this approach, it is possible to identify

significant changes in gene expression between two samples regardless of fold-change.

A typical aim in the analysis of microarray data is to compare the expression of various genes between conditions, samples or experiments for the identification of common patterns, or clusters, of gene expression. Clustering techniques are based on similarity measures (or metrics, which indicates the degree of distance in a matrix between two vectors, or genes) to create groups of features with similar patterns. Current methodologies can be divided into two main categories: supervised approaches, or analysis to determine genes that fit a pre-determined pattern; and unsupervised approaches, which characterizes datasets without the prior knowledge or input from a training dataset (Butte 2002). Among the unsupervised techniques, hierarchical clustering is the most commonly used analytical method. In this method, genes are organized into classes and subclasses that are represented as a single hierarchical tree, or a dendogram, whose branch lengths represent the degree of similarity between the sets. The usefulness of this approach is demonstrated by its tendency to organize genes into functional categories based on their expression, where genes that are expressed together are expected to have similar functions (Eisen *et al.* 1998). Non-hierarchical techniques for unsupervised clustering also exist. In *k*-means clustering, one can use prior information about the number of clusters to be used to categorise or classify the different genes expressed. *K*-means clustering simply partitions expression data into K groups and does not produce a dendogram, although one can be constructed later by a hierarchical procedure. One way to derive the appropriate number of clusters can be through principal component analysis (PCA), a mathematical decomposition technique that searches for the number of correlated patterns of gene expression by picking out the most abundant themes that reoccur in a dataset (Quackenbush 2001). Another unsupervised clustering approach is self-organizing maps (SOM). While in *k*-means the number of clusters to fit the data into are determined by the user, in SOM the shape and size (represented by a rectangular grid) of a network of clusters to fit the data into are determined by the user. These clusters are called nodes. SOM initially populates its nodes, or clusters, by randomly sampling the data, and then refines the nodes in a systematic fashion (Tamayo *et al.* 1999). SOM differs from *k*-means in that it automatically provides some information on the similarity between nodes, as nodes with similar expression patterns will be located close to each other on the grid. In the case of supervised

clustering methods, such as support vector machines (SVMs), prior information about the pattern of transcription is used to train the SVM to determine whether a transcript belongs to a particular class (Pollock 2002).

When using clustering techniques, there are many similarity measures and clustering algorithms and each can reveal different aspects of the data. Thus, it is best to analyse the data using several methods rather than just one, and investigators must use judgement to decide whether the clusters generated make biological sense. Regardless of the analytical method used to interpret the data, the results and predictions must be further tested by other more traditional techniques.

1.4.4 Current limitations of microarray technology

The existence of several platforms for measuring gene expression makes consistency and reproducibility across technologies an important factor. In addition to the different probe types (cDNA or oligonucleotides), these two main platforms differ in array manufacturing and design, probe content, probe deposition technology, labelling and hybridization protocols, scanning, and data handling (Barrett & Kawasaki 2003). Being able to use data interchangeably across platforms would be very beneficial, as this would potentially reduce the need to duplicate experiments. However, it is still a matter of controversy whether it is indeed possible to directly compare gene expression results from different microarray platforms. For example, Kuo *et al* (Kuo *et al.* 2002) analysed the expression of 2895 genes across 56 cell lines from the National Cancer Institute's standard panel of 60 cancer cell lines (NCI-60) from experiments performed with Stanford-type cDNA microarrays and Affymetrix oligonucleotide microarrays. They observed a poor correlation between the two tested platforms in all measurements of similarity, including cluster analysis. The authors do point out though, that these experiments were carried out in two different laboratories, at different times and using different materials and protocols, possibly explaining the discrepancies observed. Similar results were obtained by other groups, who reported large discrepancies in fold-change calculations and varied detection sensitivity between the two platforms (Kothapalli *et al.* 2002,Li *et al.* 2002b). On the other hand, Yauk *et al* (Yauk *et al.* 2004) examined the reproducibility and sensitivity in six microarray technologies, comprised of two cDNA arrays, three short oligonucleotide arrays and one long oligonucleotide array.

They found low levels of variance within each of the platforms as well as between platform pairs, and concluded that, given high-quality arrays and appropriate normalization, the main factor determining variance is biological rather than technological. Lee *et* al (Lee *et al.* 2003) developed a "mutual validation algorithm" with which (using the NCI-60 cancer cell lines) they identified a consensus gene expression data set using both cDNA and oligonucleotide arrays. The authors further suggested that researchers use both platforms to mutually validate a dataset, with the argument that if a common number of genes is identified as differentially regulated using two very different protocols, the confidence in the results is greatly increased. Nevertheless, the above observations highlight the need for a standardized microarray system and suggest that data must be further validated using other gene expression techniques such as Northern blotting, RNase protection or PCR.

As discussed above, variability across experiments is a major limitation of microarray technology, and the type of platform used in an array experiment is an important source of variation in microarray studies. However, other factors may also influence the outcome of the experiment. These include: methods for processing tissue and RNA isolation; efficiency of the reverse transcription step for cDNA generation; array spotting errors; hybridization and washing conditions; non-specific hybridization; background; and efficiency in dye incorporation (Schuchhardt *et al.* 2000). Various attempts are being made to minimize such problems, including many different statistical and normalization techniques. Furthermore, in order to address the problem of lack of standardization, the Microarray Gene Expression Data (MGED) Society has proposed a standard Minimum Information About a Microarray Experiment (MIAME) (Brazma *et al.* 2001). This standard is intended to ensure that the information needed for interpretation and independent verification of experimental results is publicly available, and it consists of 6 parts: (i) experimental design; (ii) array design, (iii) samples, (iv) hybridizations; (v) measurements; and (vi) normalization controls. MIAME has been embraced by the scientific community, and many journals now require compliance with MIAME for submission. Other limitations of microarray experiments include sample preparation methods, which require relatively large quantities of RNA, limiting the study of discrete cell types or tissues in complex structures; and the cost and infrastructure required for array experiments, which can act as an obstacle for many laboratories and may result in studies with less than satisfactory experimental and statistical designs.

Despite such limitations, microarray technology is rapidly progressing towards improved reproducibility and standardization, and has already provided a wealth of information and been successfully applied to a wide range of topics including toxicity profiling, drug screening, and genomic characterization of disease (Blohm & Guiseppi-Elie 2001,Li *et al.* 2002b).

1.4.5 Applications of microarray technology

One of the most widely used research applications of microarrays is gene expression profiling of tumours. Transcriptional profiling makes it possible to systematically explore global transcription patterns associated with malignant transformation and to correlate these with clinical phenotypes. The central goal of these studies is the early detection of disease pathology, diagnosis including class and outcome prediction and identification of causal genes themselves. Microarray has been shown to identify candidate molecular markers for ovarian cancer (Welsh *et al.* 2001), identify prognostic markers for prostate cancer (Dhanasekaran *et al.* 2001b) and predict subtypes of cutaneous melanoma (Bittner *et al.* 2000). Furthermore, transcriptional profiling allowed researchers to differentiate two otherwise indistinguishable types of non-Hodgkin's lymphoma by analysing the expression of 17,856 genes in specimens of patients suffering from diffuse large B-cell lymphoma (Alizadeh *et al.* 2000). In lung cancers, obvious gene expression differences were found across tumours of the different cell types: small cell, large cell, squamous cell and adenocarcinoma (Garber *et al.* 2001). Gene expression profiling has also aided in the study of temporal gene expression, for example in the identification of genes periodically expressed during the cell cycle and how their expression is altered in tumours (Whitfield *et al.* 2002). When coupled with transgenic model organisms, microarrays can provide valuable insight into human disease, as demonstrate by Clark *at al* (Clark *et al.* 2000). They compared poorly metastatic and highly metastatic melanoma cell lines isolated by *in vivo* selection in mice, and detected a strong correlation between the severity of tumour metastasis and the expression of the small GTPase RhoC. Another important metastasis study was carried out by Yang *et* al (Yang *et al.* 2004). Using microarray analysis, the group compared the expression profiles of four cell lines, all derived from the same spontaneous mouse mammary gland tumour, and which displayed distinct

characteristics of the key phases of cancer progression when injected into the mammary glands of mice. They found that the transcription factor Twist, a key embryonic regulator, was highly upregulated in the primary tumours that produced cells capable of penetrating blood vessels (intravasation) and of forming metastatic nodules in the lung. The authors then used RNA interference to deplete Twist in metastatic primary tumour cells and showed that this substantially reduced intravasation, thereby decreasing the amount of tumour cells in blood circulation and the number of lung metastases. Thus, by further testing the correlation between Twist expression and metastasis found by microarray data analysis, the authors were able to determine a causal role for Twist in tumour metastasis. Gene expression profiling has also been used to identify changes in gene expression associated with specific genes known to be involved in cancer cell signalling. Schulze *et al* (Schulze *et al.* 2004) measured the expression profile of 6,000 unique genes in a Raf-inducible cell system. Raf is a downstream effector of Ras, the dominantly acting oncogene that is implicated most frequently in human cancers, and is responsible for the phosphorylation of MEK1 and MEK2, which in turn phosphorylate ERK1 and ERK2. As such, Raf is a key player in the MAPK signalling pathway. The investigators found 135 genes that were significantly changing following Raf activation, and using a MEK-specific inhibitor, they showed that the Raf-mediated changes in gene expression are dependent on its ability to activate MEK. In addition, they showed that changes in expression of about 40% of the Raf-induced genes can be blocked by inhibition of EGFR, reflecting the large role Raf activation plays in inducing an EGF autocrine loop. Finally, many recent studies in tumour profiling have concentrated on breast cancer, and this is discussed in more detail in the next section.

In contexts other than tumour profiling, microarrays have been used for large-scale genotyping. For example, single nucleotide polymorphisms (SNP) from over 64,000 probes have been analysed using high-density oligonucleotide arrays (Fan *et al.* 2000), and by analysing 1494 human SNP loci by microarray, loss of heterozygosity was shown to occur during tumourigenesis (Lindblad-Toh *et al.* 2000). Microarrays can also be useful in the validation of therapeutic targets when combined with gene silencing of targets through RNA interference. Indeed, the effectiveness of this approach was demonstrated in a study whereby the effect of *Rb1* knockdown was analysed, identifying downstream targets of Rb with roles in DNA replication and repair, mitosis and apoptosis (Semizarov *et al.* 2004). Alternatively,

the overexpression of a candidate therapeutic target gene offers an important tool for defining its biological function. Fambrough *et al* (Fambrough *et al.* 1999) characterized the signalling pathways of PDGFβ receptor and FGF receptor 1 using a gene transfection approach in combination with arrays, and showed that activation of differentially engineered mutant PDGF and FGF receptors results in highly overlapping patterns of mRNA expression. In a similar context, new technologies are emerging whereby living cells cultured on a glass slide take up different cDNAs that are printed at defined locations (Ziauddin & Sabatini 2001). This remarkable technology makes it possible to screen the effects of overexpressing many genes in a single experiment. In the study carried out by Ziauddini & Sabatini, 192 different cDNAs were used for the 'transfected cell microarray', and the authors were able to identify gene products involved in tyrosine kinase signalling, apoptosis and cell adhesion. Chromatin immunoprecipitation followed by array hybridization has also been reported, enabling the identification of transcription factor binding sites across the entire genome and the construction of transcriptional networks (Iyer *et al.* 2001). This analysis identified distinct functional gene clusters that are controlled by specific cell cycle transcription factors in yeast. It is also possible to use microarrays to identify splice variants. For example, Hu *et al* (Hu *et al.* 2001a) used Affymetrix arrays to screen 1600 rat genes across 11 tissue types, identifying tissue-specific splice variants by observing probe-specific differences in hybridization across tissues. Furthermore, single cell gene expression assays have been developed whereby it is possible to simultaneously visualize the expression of many genes inside a single cell (Levsky *et al.* 2002), allowing a more precise study of complex cellular interactions.

The examples above highlight the usefulness of microarray technology and its numerous possible applications. They also show that the combination of microarray technology and other traditional molecular biology techniques offers a powerful tool for researchers. Moreover, other new technologies based on microarray, such as tissue and protein arrays, are emerging as useful tools in the study of cancer and other human diseases. Thus, microarray studies will greatly enhance our knowledge and understanding in a variety of areas in molecular biology.

1.4.6 Microarray and the study of breast cancer

Microarray technology in the study of breast cancer is complicated due to the great clinical, histological and biochemical diversity of this disease. Indeed, hierarchical clustering of microarray data derived from measurements of expression of 8,000 genes within the panel of NCI-60 human cancer cell lines showed that while cells of the same tissue of origin grouped together, breast cancer cell lines have a more heterogeneous gene expression pattern and did not form an independent cluster (Ross *et al.* 2000). Nonetheless, microarray analysis has been successfully used to predict the clinical status of breast cancers, such as the oestrogen receptor status (West *et al.* 2001), thereby allowing the most suitable therapeutic approach to be chosen for a specific tumour. It has also been demonstrated that gene expression profiling can differentiate tumour samples with mutations in BRCA1 and BRCA2 tumour suppressor genes when compared to sporadic cancers lacking these mutations (Hedenfalk *et al.* 2001). This was interesting because although mutation in either gene leads to breast cancer, they show striking differences in their gene expression patterns, indicating that their mechanisms of induction of malignant transformation are not the same.

Gene expression profiling of breast tumours also allows the identification of markers that may be important in predicting patient prognosis, potentially enabling clinicians to decide which patients may need to be subjected to adjuvant therapy. Sorlie *et al* (Sorlie *et al.* 2001) showed that breast tumours can be subdivided into classes, such as basal-like tumours, ErbB2-positive tumours and luminal ER-positive tumours based on clustering of their gene expression patterns. Moreover, these gene expression profiles could be used as predictors for overall and relapse-free survival, with basal-like and ErbB2-positive subtypes being associated with the shortest survival time. Importantly, the authors further demonstrate that ER-positive tumours can be classified into distinct subgroups with different patient outcome, raising the question of whether ER is indeed a reliable prognostic marker. Another important study confirming the usefulness of microarrays as a prognostic tool was carried out by van't Veer *et al* (van't Veer *et al.* 2002). This study analysed primary tumours from 117 patients (all less that 5cm in diameter, from lymph node-negative patients who were less than 55 years of age), and carried out a supervised analysis in which individuals were separated into 2 groups: those who developed metastasis within 5

years (bad prognosis) and those that were metastasis-free for longer than 5 years (good prognosis). They identified a set of 70 genes whose expression correlated with this parameter, and showed 81% accuracy when tested in a "leave-one-out" cross-validation analysis of the training set, and 89% accuracy on a test set. These reporter genes were further tested on 295 young breast cancer patients, and were shown to outperform clinical parameters currently used in determining disease outcome, such as lymph node status, tumour size, pathological stage and histological type of tumour (van de Vijver *et al.* 2002). This suggests that a gene expression profile is a far better predictor of breast cancer outcome than any other criteria and may be a powerful tool for determining which patients would benefit the most from adjuvant therapy, sparing the remaining patients from the toxic side effects of this type of treatment. Indeed, if the prognostic value of this approach can be further validated and confirmed, the expression profiling classifier would result in about a four-fold decrease in the number of patients receiving adjuvant therapy unnecessarily (Caldas & Aparicio 2002).

Most breast cancers are sporadic and develop through the accumulation of more than one genetic lesion. The study of breast cancer in transgenic mice offers an invaluable tool to study mammary tumour progression caused by a specific initiating event, for example through targeted overexpression of a particular oncogene. Using well characterized mouse models of human breast cancer, Desai *et al* (Desai *et al.* 2002) used microarrays to determine the differences in gene expression between normal mammary gland and mammary tumours initiated by transgenic overexpression of six oncogenes, including c-Myc, Neu (ErbB2) and Ha-Ras. The authors showed that the tumours were highly similar in their gene expression profiles irrespective of the initiating oncogenic event, although they differed significantly from normal mammary gland. Further statistical manipulation identified genes and potential pathways uniquely perturbed by the specific initiating oncogenic event, subdividing the mouse model into three groups with specific patterns of gene expression, which may potentially facilitate the development of oncogene-specific therapeutics.

Drug resistance is a major obstacle to successful chemotherapy, and therefore obtaining a molecular profile of anticancer drugs in tumour samples or cancer cells by microarray technology would be critical to predict chemotherapy response. Expression patterns of breast cancer patients undergoing doxorubicin-based

chemotherapy were analysed after the first cycle and gene expression following treatment was correlated with patient outcome (Sotiriou *et al.* 2002). The expression profiles of doxorubicin-treated normal and doxorubicin-resistant MCF-7 breast cancer cells were also compared, and the results showed that transient treatment with this chemotherapy drug altered the expression of a diverse group of genes in a time-dependent manner (Kudoh *et al.* 2000).

Although gene expression profiling is sensitive enough to detect even low-fold changes in expression levels, most studies have been performed using heterogeneous tumour tissues, which contain variable amounts of non-tumour cells as well as tumour cells, and it may be that some of the genes recognized as significantly changing are not truly representative of oncogenic changes. To solve this problem, researchers have used microdissected tumour tissue (Maitra *et al.* 2001). Using a combination of laser capture microdissection and DNA microarrays, Ma *et al* attempted to identify particular gene expression profiles that could be correlated with the different pathological stages of breast cancer (Ma *et al.* 2003). They found no consistent changes in gene expression that could be associated with any of the three pathological stages examined, these being atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC), representing the pre-malignant, pre-invasive and invasive stages of breast cancer, respectively. This suggests that significant changes in gene expression occur at the earliest phenotypically recognized stage of tumorigenesis. However, the authors were able to identify distinct gene expression patterns associated with different tumour grades (i.e. the degree of malignancy rather than the stage of malignancy, based on cellular proliferation, differentiation state and on hormone receptor and ErbB2 expression levels) as well as a small subset of genes with quantitative expression levels that correlated with advanced tumour grade and with the transition between DCIS and IDC. These results suggest that the transcriptional program that drives cancer cells through to an advanced tumour grade may also confer invasiveness.

Many microarray studies have been carried out in breast cancer cell lines. For example, Perou *et al* (Perou *et al.* 1999) have studied the gene expression profile of human mammary epithelial cells following a set of treatments, including TGF-β1, interferon (IFN) and EGF treatment, 100% confluency and senescence. They identified a "proliferation-associated cluster", which included many genes involved in cell cycle progression (e.g. *CDC47, MCM3, MCM6*, cyclin B1 and Ki-67). Their

expression was reduced following treatment of cells with factors known to be associated with inhibition of proliferation, such as IFNα, IFNγ or TGF-β1 stimulation, withdrawal of EGF, confluence and senescence. This data was then compared to breast tumours, tissues and breast cancer cell lines, and it was found that gene expression patterns in breast cancer could be related to this proliferation cluster, with this set of genes being highly expressed in rapidly proliferating cell lines and in a subset of tumour specimens.

A number of microarray studies using cell lines have specifically looked at the effects of ErbB2 overexpression. In a study of 145 breast cancer samples, Bertucci and colleagues (Bertucci *et al.* 2004) analysed the expression of 9038 clones (representing 5125 known genes) and identified an ErbB2 "gene expression signature". This signature consisted of 37 clones corresponding to 36 unique sequences, 29 of which were characterized genes, which successfully separated ErbB2-positive and ErbB2-negative cell lines as well as breast tumours. This gene expression signature included genes located in the same chromosomal region as ErbB2 (17q12) which are likely to be co-amplified with ErbB2, such as *GRB7* and *PPARBP*. Other overexpressed genes included the integrins *ITGA2, ITGA2B* and *ITGB3* as well as *GATA4, MAP2K6, CSTA* and *MKI67*. In this study, only five genes were downregulated in the ErbB2 positive signature, including *ESR1, SCUBE2,* and *CELSR2*. Whilst several other studies have reported overlapping transcriptional profiles associated with ErbB2 overexpression, discordant changes were reported (Wilson *et al.* 2002,Andrechek *et al.* 2003,Kumar-Sinha *et al.* 2003). Such discrepancies are likely due to the different cell types used, different data analysis techniques adopted and variations inherent to microarray technology.

In the context of this thesis, the study by White *et al* (White *et al.* 2004) and Mackay *et al* (Mackay *et al.* 2003) are of particular interest. They used microarrays to study the effects of ErbB2 overexpression using the normal mammary luminal epithelial cell line HB4a and its ErbB2-overxpressing clone C3.6, the same cell system used in this study (described in section 1.3). Mackay *et al* also analysed an additional HB4a-derived clone, C5.2, which expresses even higher levels of ErbB2 than the C3.6 cells. They identified 61 genes that were significantly up- or downregulated in response to ErbB2 overexpression, 32 of which were confirmed by PCR, and two of which were confirmed using tissue arrays containing a mixture of ErbB2-positive and ErbB2 negative primary breast tumours. White *et al* looked at

gene expression in HB4a and C3.6 cells, and further stimulated these cells with HRGβ1 to investigate signalling events downstream of ErbB2/ErbB3 heterodimers. They correlated changes in gene expression with the transformed characteristics of these cell lines with respect to autocrine signalling, cell cycle progression and cellular adhesion. A number of common genes between the two studies were found to be differentially regulated by ErbB2 overexpression, including *S100P*, *CPS1* and *VIM* (upregulated) and *IGFBP3*, *FN1* and *SPARC* (downregulated).

In conclusion, microarray studies have greatly enhanced our understanding of tumour biology. Scientists are able to distinguish tumour types, identify target genes and pathways, predict patient prognosis, define optimal therapeutic intervention and assess pharmacodynamic responses using gene expression profiling (Liu *et al.* 2003). In other cases, microarray analysis has identified novel genes or pathways involved in cancer, and although this technology may not provide an understanding of the molecular mechanisms that result in cancer, it can certainly provide several testable hypotheses. The current challenge lies in overcoming the limitations of the technology, making data from different laboratories comparable and array experiments more practical and cost effective. New technical solutions are being developed to improve current instrumentation and to fulfil the requirements for "second-generation" microarrays, including methods for label-free detection systems, automated flow-through systems for time-resolved hybridization and powerful data processing software (Blohm & Guiseppi-Elie 2001). Thus, there is little doubt that microarrays will have a major impact on cancer research, allowing researchers to decipher the complex network of interactions of genes involved in oncogenic transformation.

1.5 Aims of this Study

Microarray technology has evolved considerably over the past few years, and major improvements in the technique as well as in data analysis strategies have been developed, providing powerful tools for large scale gene expression analysis of complex cellular events. The aim of the present study was to understand the role of ErbB2 overexpression in cellular transformation and in the development of cancers, particularly breast cancer. As such, cDNA microarray technology was used to search for downstream changes in gene expression as a consequence of ErbB2

overexpression and of ErbB-specific growth factor signalling, and to potentially identify new molecular markers of ErbB2 in breast cancer. ErbB2 is thought to play a key role in the control of complex signalling events through interactions with other ErbB family members and cross-talk with other signalling pathways. ErbB2 overexpression is therefore expected to affect several known key cellular processes as well as previously uncharacterised cellular functions, which would be important to assess in the attempt to identify new targets for breast cancer therapy.

Microarray experiments were performed using a model cell system comprised of the parental cell line HB4a and its ErbB2 overexpressing derivative, C3.6 in order to obtain a general view on the complex mechanisms of ErbB2-dependent signalling and breast cancer development. An additional aim of this study was to understand signalling pathways downstream of different ErbB receptor dimers and the specificity/overlap in these responses. To examine this, microarray experiments were carried out where both HB4a and C3.6 cells were stimulated over a time course with growth factors specific for different family members, namely EGF and HRG. Thus, changes in genes expression by growth factor treatment in an ErbB2- and time-dependent fashion can be identified, enabling a deeper understanding of the signalling mechanisms of ErbB signalling in the context of ErbB2 overexpression. To the best of our knowledge, this is the first study to simultaneously address ErbB2 overexpression as well as ErbB signalling through different receptor dimers using a model of ErbB2-overexpressing breast cancer and a paired control. This microarray analysis and a description of the genes found to be differentially expressed in response to ErbB2 and/or growth factors are described in Chapter 3. Although various statistical methods are available to increase the confidence in the results obtained from microarray experiments, it is essential to validate the findings derived from analysis through other techniques. This is addressed in Chapter 4, where a number of genes of special interest are investigated using real-time PCR to confirm the changes in mRNA levels. Corresponding changes in the expression of protein products from selected differentially expressed genes were also addressed in Chapter 4. Finally, in order to understand the impact of the differential regulation of selected potential ErbB2 markers, it was of great interest to further characterize a subset differentially expressed genes in the context of cancer cell biology. Chapter 5 describes the functional characterization of potential targets of ErbB2 overexpression, namely members of the interferon signalling pathway, using a number of traditional

biochemical and cell biology approaches. The effect of ErbB2 overexpression was investigated with particular emphasis on the cross-talk between signalling downstream of ErbB family members and the interferon signalling pathway.

Chapter 2: MATERIALS & METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Company Ltd., unless otherwise stated.

2.1 Cell Culture

HB4a, C3.6 and other clones derived from the HB4a cell line, were obtained from Michael O'Hare (Ludwig Institute for Cancer Research). These cell lines were generated as described (Harris *et al.* 1999). Cells were maintained in 1640 RPMI media containing 10% (v/v) foetal calf serum (FCS), supplemented with L-glutamine (2 mM), streptomycin (100 μg/mL), penicillin (100 IU/mL) (all purchased from Gibco-Invitrogen Corp.), insulin (5 μg/mL) and hydrocortisone (5 μg/mL) in tissue culture dishes at 37°C and 10% CO_2 in a humidified incubator. MCF-7 cells were grown in DMEM-F12 media (Gibco-Invitrogen Corp.) supplemented with 10% (v/v) FCS, streptomycin (100 μg/mL) and penicillin (100 IU), in tissue culture dishes at 37°C and 5% CO_2 in a humidified incubator. All cells were passaged when they reached 70-80% confluence by trypsinisation according to standard procedures.

Stocks of luminal epithelial cells were stored in liquid nitrogen and new batches of low passage cells frequently thawed for usage to avoid potential genetic drift and clonal selection which can occur during prolonged passaging of cells. For freezing, trypsinised cells were pelleted and resuspended in 10% (v/v) DMSO in 90% (v/v) FCS. Cells were slowly taken to –70°C in a propanol container and then transferred to liquid nitrogen for long-term storage. Rapid thawing was carried out in a 37°C water bath and excess DMSO was removed by centrifugation prior to plating. Cells were allowed to recover for three passages prior to sampling.

2.1.1 Growth factor, interferon and inhibitor treatment

Prior to growth factor treatment, HB4a and C3.6 cells were serum-starved in 0.1% (v/v) FCS in 1640 RPMI media supplemented with L-glutamine (2 mM), hydrocortisone (5 μg/mL) and antibiotics for 24 to 48 hours. MCF-7 cells were serum-starved in DMEM-F12 media containing 0.1% FCS and antibiotics. Cells were then treated for various periods of time with either 1 nM (6 ng/mL) epidermal

growth factor (EGF) or 1 nM (8 ng/mL) Heregulinβ1 (HRG) (both R&D systems). For the interferon (IFN) studies, cells were stimulated with IFNβ (PBL Biomedical Laboratories) or IFNγ (R&D Systems), both at 1000 IU/mL. The specific ErbB tyrosine kinase inhibitor AG1478 (Biosource) was used at 5 μM, a concentration which can inhibit tyrosine phosphorylation of both EGFR and ErbB2 receptors and abrogate downstream activation of the MAPK and PI3K pathways (Timms, unpublished data). The MEK inhibitor PD098059 (Calbiochem) was used at 10 μM (Dudley *et al.* 1995). Inhibition of the proteasome was carried out using 1 μM PS341 (Millenium Pharmaceuticals, Inc.) (Adams *et al.* 1999). Inhibition of protein synthesis was achieved using 10 μg/mL cycloheximide (CHX).

2.2 Microarray Sample Preparation & Data analysis

2.2.1 Microarrays used in the present study

The cDNA microarrays used in this study were constructed at the Wellcome Trust Sanger Institute as part of the LICR/CRUK Microarray Consortium. Each microarray contains a redundant set of 9932 PCR-derived, sequence verified cDNA clones representing approximately 6,000 unique genes. The array versions used in the present study were the Hver1.2.1 and Hver1.3.1, both of which contain identical elements representing the same set of genes. Data files containing the annotation and gene lists can be found on the Institute's website*. The annotation data file used in this study was Hver1.3.1 version 33. Further information including array production pipeline, updated gene annotation and full description of array preparation can be found on The Wellcome Trust Sanger Institute Microarray Facility homepage†.

The microarray experimental design used in this study was the reference design, whereby each sample studied is co-hybridized with a common reference sample present in all the arrays. The reference sample used here was a kind gift from Dr. Alan Mackay (Institute of Cancer Research, London, UK) and consisted of total RNA pooled from the BT474 breast tumour cell line and two grade III invasive ductal breast carcinomas. In addition to the reference design, the "dye-flip" approach

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* http://www.sanger.ac.uk/Projects/Microarrays/informatics/hver1.3.1.shtml

† www.sanger.ac.uk/Projects/Microarrays/

was used to minimize dye-specific bias, whereby two replicate samples are labelled with Cy3 and the reference sample with Cy5 and two replicates labelled in reverse. For the 14 experimental conditions studied (two cell lines, two growth factor and four time points), a total of 56 hybridizations were performed. Further information on this experimental design is given in Chapter 3.

2.2.2 Sample preparation

When preparing and handling RNA samples, great care was taken in order to avoid contamination with RNases. Disposable gloves were worn at all times and frequently changed. Sterile, disposable plasticware and filtered pipette tips free of RNase dedicated exclusively to handling RNA samples were used. Glassware was baked at 150°C for 4 hours prior to usage. All solutions used in RNA sample preparation were prepared using DEPC-treated water. DEPC (Diethylpyrocarbonate) is a strong inhibitor of RNases and it is commonly used at a concentration of 0.1% (v/v) to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC water was made by adding 1 mL of DEPC to 1 L of water, incubating overnight at room temperature and then autoclaving to remove traces of DEPC. In addition, the RNase inhibitor RNasin (Promega) was used to treat freshly isolated total RNA prior to storage at -70°C, at a concentration of 1 unit of RNasin per μL of sample.

2.2.2.1 RNA extraction

Cells were grown to 60% confluency on 15 cm plates, serum-starved and stimulated as described above. For microarray experiments, a total of 14 experimental conditions were analysed. HB4a and C3.6 cells were left un-stimulated (control samples) or treated with EGF or HRG for 4, 18 and 24 hours. Total RNA was extracted using TRIZOL reagent (Gibco-Invitrogen Corp) according to the manufacturer's instructions. Cells were washed twice with phosphate-buffered saline (PBS) and 5 mL of TRIZOL was added to each plate. Plates were incubated at room temperature for 5 minutes. Using a scraper, samples were collected into a tube containing 1 mL chloroform, shaken vigorously for 30 seconds to homogenize samples, and incubated for a further 5 min at room temperature. Samples were

centrifuged for 15 min at 12000 g at 4°C. Following centrifugation, the mixture separates into a lower organic and an upper aqueous phase. RNA remains exclusively in the aqueous phase. This phase was then transferred to a fresh tube and the RNA was recovered by precipitation with isopropyl alcohol. To achieve this, the RNA aqueous phase was mixed with 2.5 mL isopropanol, incubated at room temperature for 10 min and centrifuged for 10 min at 12000 g at 4°C. Supernatant was discarded and RNA pellet washed once with 70% ethanol. The pellet was air-dried, re-dissolved in 1 mL DEPC water and incubated at 56°C for 10 min. RNA was quantitated using a standard spectrophotometer at 260 nm wavelength, aliquoted into desired amounts for downstream applications and stored at -70°C in 75% ethanol. RNA integrity was verified by agarose gel electrophoresis and bands visualized using ethidium bromide. RNA extraction was carried out twice for each condition being studied. Each extraction procedure yielded enough RNA for two microarray experiments. Thus, a total of four replicates for each sample were prepared for microarray analysis. RNA extraction was carried out using freshly seeded cells for real time PCR experiments.

2.2.2.2 Reverse transcription and Cy dye labelling

Generation of Cy dye-labelled cDNA samples was carried out following Protocol 5 found on the Sanger Centre microarray facility homepage[‡]. 25 μg of total RNA was used to produce labelled cDNA by anchored oligo(dT)-primed reverse transcription with Superscript II Reverse Transcriptase (Invitrogen Life Technologies) in the presence of Cy3- or Cy5-dUTP (Amersham Pharmacia). Briefly, total RNA (see above) was precipitated by adding 1/40 of the sample volume of 3 M sodium acetate (pH 5.2), incubating at -70°C for 30 min and spinning samples down at 12,000 rpm for 10 min. RNA pellets were washed once in 70% ethanol and resuspended in 15.4 μL DEPC water containing 2.5 μg oligo(dT) primer. This mixture was incubated at 70°C for 10 min and snap-chilled on ice. A reaction mix containing 6 μL 5X first strand buffer (supplied with the Superscript II kit), 30 μL of 0.1 M DTT, 0.6 μL dNTP mix (composed of 25 mM dATP, dTTP and dGTP and 10 mM dCTP), 3 μL Cy3- or Cy5-dCTP and 2 μL Superscript II were added to the

[‡] http://www.sanger.ac.uk/Projects/Microarrays/arraylab/protocol5.pdf

resuspended RNA. Samples were mixed and incubated in the dark for 2 hours at 42°C. Following the reverse transcription reaction, 1.5 µL of 1 M sodium hydroxide (NaOH) was added to samples which were incubated at 70°C for 20 min to hydrolyse any RNA that had not been reverse transcribed. 1.5 µL of 1 M hydrogen chloride (HCL) was finally added to neutralize the reaction.

Unincorporated Cy dye was removed using Autoseq-50 Columns (Amersham Pharmacia). Cy3- and Cy5-labelled samples to be co-hybridized were then mixed together and co-precipitated with 8 µg of human Cot1 DNA (Invitrogen Life Technologies) and 8 µg poly(dA) DNA (Sigma-Aldrich) to block repetitive DNA sequences that can affect hybridization to the array. After precipitation, the labelled cDNA pellet was re-suspended in hybridization buffer (4X SSC, 5X Denhardt's solution, 50 mM Tris-HCl pH 7.6, 0.1% sarkosyl, 49% formamide).

2.2.3 Array hybridization, scanning and image processing

Microarray slides were soaked in hybridization buffer and incubated at 47°C for 2-4 hours and then centrifuged for 1 min prior to sample hybridization. Without letting them dry, the microarray slides were lowered onto coverslips layered with the Cy dye labelled cDNA samples. Slides were placed in a humidified chamber and allowed to hybridize overnight at 47°C. Slides were then washed twice in 2X SSC, four times in 0.1X SSC/0.1% SDS, twice in 0.1X SSC and then dried by centrifugation before scanning. Fluorescent images of hybridized microarrays were captured using the GSI Luminomics 4000 scanner with ScanArray software (Packard Biosciences/PerkinElmer). Images were quantitated and background subtracted using GSI Luminomics Quantarray 3.0 software (Packard Biosciences/PerkinElmer). The background-corrected fluorescence intensity measurements from each experiment were transferred into the GeneSpring version 6.1 software (Silicon Genetics) for data normalization using the LOWESS regression technique (Yang *et al.* 2002). Image acquisition, quantitation and data normalization are discussed in more detail in Chapters 1 and 3.

2.2.4 Microarray data analysis

The excel add-in software SAM (significance analysis of microarrays) (Tusher *et al.* 2001) was used to identify statistically significant changes in gene expression across the samples studied here. SAM works by assigning a value for each gene on an array which describes how likely it is that the gene is expressed differently between two populations based on the spread of values for the replicates. The percentage of genes identified by chance, or the false discovery rate (FDR), is calculated based on permutations of the measurements and is presented as a q-value for each gene in the final list of significant genes. SAM allows control of the FDR by changing the delta parameter, which adjusts the q-value threshold. The q-value represents the chance that the gene is a false positive, and is the lowest FDR where the gene is considered significant. In other words, the q-value is a measure of the probability that a gene which appears to be differentially expressed is not actually differentially expressed at all and is similar to the *p* value in a Student's t-test. In this study, a FDR threshold of 3% was used. SAM then displays a plot representing each gene based on its score in the real distribution compared to random distributions, where differentially expressed genes are represented outside of the two slope lines (Figure 2.1).

SAM analysis was carried out using $\log_2$-transformed ratios using the two-class unpaired response format (for more information, refer to the SAM user's manual[§]), so only two samples are compared at one time. Thus, to measure changes in gene expression values, 28 comparisons were carried out where HB4a and C3.6 cells were compared directly at each time point for both growth factors, and for each individual cell line, all time points were compared against each other.

Once significantly changing genes were identified, data was visualised using different clustering techniques. Hierarchical and *k*-means clustering were performed using the TIGR Multiexperiment Viewer (MeV) software version 2.2 (The Institute for Genomic Research) (Saeed *et al.* 2003). Normalized expression levels for each gene were used to calculate ratios representing the relative expression of a particular gene relative to an assigned control. Two ratios were used in this analysis which allowed the determination of gene expression in C3.6 cells relative to HB4a as well as in growth factor treated samples relative to the un-stimulated control for each cell

[§] http://www.utulsa.edu/microarray/Articles/sam%20manual.pdf

line. These ratios are explained in Chapter 3, Section 3.4.3. Ratios were then log_2-transformed and loaded into the TIGR MeV software for clustering analysis.

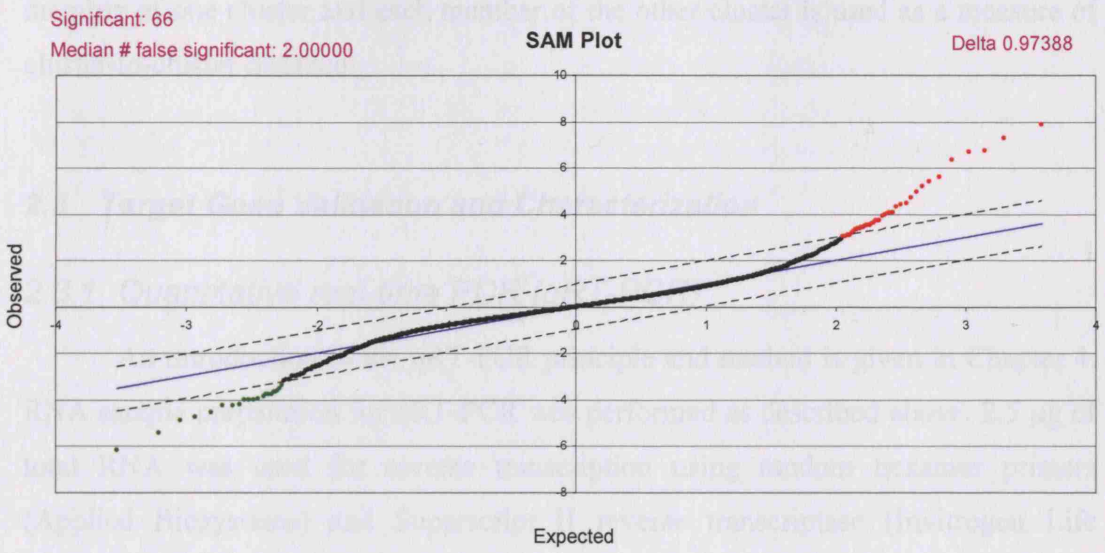

Figure 2.1: SAM plot. Example of a typical plot generated using SAM for data filtering of microarray experiments. Genes differentially regulated are represented by red and green dots. In this example, delta was set at 0.97, giving a total of 65 differentially expressed genes with 2 potential false positives (FDR=3%).

When performing clustering techniques on gene expression data, a means of measuring quantitatively if two expression profiles are similar to each other is required. It is therefore useful to consider the values that make up the expression profile for a single gene as a series of coordinates, which define a vector, and to consider the data for a microarray experiment as a matrix, where the genes define the rows, and the arrays define the columns. Thus, it is possible to use standard mathematical techniques to measure the distance between vectors and to measure their similarity (Brazma & Vilo 2000). In the present study, the distance metrics used to construct clusters of similarly expressed genes was the Euclidean distance, which measures the absolute distance between two expression vectors in space. The Euclidean distance therefore takes into account both the direction and the magnitude of the vectors. Note that because the Euclidean distance will be affected by both the

direction and the amplitude of the vectors, two genes that are co-ordinately expressed might not be seen to be similar if one has a much higher signal than the other. For hierarchical clustering, the average linkage clustering method was used to determine the distance between clusters. In this method, the average distance between each member of one cluster and each member of the other cluster is used as a measure of cluster-to-cluster distance.

2.3 Target Gene Validation and Characterization

2.3.1 Quantitative real-time PCR (qRT-PCR)

An introduction to the qRT-PCR principle and method is given in Chapter 4. RNA sample preparation for qRT-PCR was performed as described above. 2.5 μg of total RNA was used for reverse transcription using random hexamer primers (Applied Biosystems) and Superscript II reverse transcriptase (Invitrogen Life Technologies) following the manufacturer's protocol. In order to remove traces of RNA complementary to the cDNA, samples were treated with 2 units of *E. coli* RNase H (Invitrogen Life Technologies) and incubated for 20 min at 37°C. Samples were then stored at -20°C until use in qRT-PCR experiments. The primers and probes used were all obtained from Applied Biosystems as part of the "TaqMan Gene Expression Assays" (for more information, see www.appliedbiosystems.com). These gene expression assays are a comprehensive set of pre-designed gene-specific probe and primer sets. Each assay consists of two unlabelled gene-specific PCR primers and a gene-specific TaqMan probe labelled with the reporter dye 6-FAM at its 5' end and a non-fluorescent quencher (NFQ) at the 3' end. All components, which are quality control-tested, are provided in a single reaction mix. These assays have been optimized and validated using universal cycling conditions, thereby greatly reducing the time required for optimizing PCR conditions prior to carrying out the reactions. Assays for specific genes can be ordered directly from the company's website. A complete list of the assay IDs used in this study can be found in Table 2.1 below. Additional components for the qRT-PCR reaction were: 96-well optical reaction plates, optical adhesive covers and TaqMan universal PCR master mix. These were all purchased from Applied Biosystems.

Gene Symbol	ABI assay ID	Gene name
AREG	Hs00155832_m1	Amphiregulin
CTSB	Hs00157194_m1	Cathepsin B
CTSC	Hs00175188_m1	Cathepsin C
G1P2	Hs00192713_m1	Interferon, alpha-inducible protein (clone IFI-15K)
IGFBP3	Hs00426287_m1	Insulin-like growth factor binding protein 3
ISGF3G	Hs00196051_m1	Interferon-stimulated transcription factor 3, gamma 48kDa
OAS1	Hs00242943_m1	2',5'-oligoadenylate synthetase 1, 40/46kDa
S100P	Hs00195584_m1	S100 calcium binding protein P
SFN	Hs00602835_s1	Stratifin (14-3-3σ)
SOD2	Hs00167309_m1	Superoxide dismutase 2, mitochondrial
STAT1	Hs00234829_m1	Signal transducer and activator of transcription 1, 91kDa
UBE1L	Hs00163295_m1	Ubiquitin-activating enzyme E1-like
USP18	Hs00276441_m1	Ubiquitin specific protease 18
VEGF	Hs00173626_m1	Vascular endothelial growth factor
VIL2	Hs00185574_m1	Villin 2 (ezrin)
VIM	Hs00185584_m1	Vimentin
ZYX	Hs00170299_m1	Zyxin
18S	**Hs99999901_s1**	**Eukaryotic 18S rRNA (endogenous control)**

Table 2.1: Applied Biosystems TaqMan gene expression assay ID numbers.

Each well of the qRT-PCR reaction plate contained the following sample volumes: 1.25 μL primer/probe mix, 2 μL cDNA sample, 12.5 μL TaqMan Universal PCR master mix and water to 25 μL. These reactions were set up by mixing all the solutions above, excluding the cDNA, in an eppendorf tube containing the appropriate volume for the number of wells desired and mixing the contents well. cDNA was added separately to each well. The reaction plate was covered with adhesive covers and briefly spun in a centrifuge to remove any residual sample from the tube walls and to eliminate air bubbles from the solution. Reactions were then run on the ABI Prism 7700 sequence detection system (PerkinElmer/Applied Biosystems) under the following cycling conditions: 95°C for 10 minutes (enzyme activation) and then 40 repeating cycles of 95°C for 15 seconds (denaturing) and 60°C for 1 min (annealing/extension). Data analysis was carried out by viewing the amplification plot for the entire plate, setting the baseline and threshold values to obtain Ct values above background and transferring data into excel to calculate

relative gene expression. Relative gene expression quantitation was obtained using the standard curve method using the HB4a control samples as the calibrator. Standard curves were constructed by carrying out serial dilutions of the calibrator sample, and were constructed for each gene analysed as well as for the 18S endogenous control. Further details and explanation of the standard curve method of qRT-PCR quantitation are given in Chapter 4, Section 4.2.1, and can also be found at the Applied Biosystems user bulletin #2**.

2.3.2 *Western blotting*

Cells were serum-starved and stimulated as described above. Cell lysis was always carried out on sub-confluent cells (70-80% confluency). Cells were washed twice in ice-cold PBS and lysed in NP40 lysis buffer (50 mM HEPES; 150 mM NaCl; 1% NP40; 1 mM EDTA) supplemented with protease inhibitors and phosphatase inhibitors (100 μg/mL AEBSF, 17 μg/mL aprotinin, 5 μM BpVphen, 5 μM fenvalerate, 1 μg/mL leupeptin, 1 μM okadaic acid, 1 μg/mL pepstatin, 2 mM sodium orthovanadate). Cells from a 10 cm plate were normally lysed in 500 μL of lysis buffer and scraped on ice. Lysates were left 20 min on ice and cell debris was cleared by centrifugation at 14,000 rpm for 10 min at 4°C.

Protein concentrations were determined using a Bradford microtitre plate assay. 2 μl of total cell lysate was used and mixed with 200 μL of Coomassie protein assay reagent (Pierce-Perbio). Absorbance readings were taken at 595 nm on a microtitre plate spectrophotometer. Standard curves were determined using dilutions of bovine serum albumin (BSA) and samples were assayed in triplicate. Cell lystates were then reduced and denatured in sample buffer (50 mM Tris pH 6.8, 10% (v/v) glycerol, 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 2% β-mercaptoethanol) and boiling for 5 min at 100°C.

For western blotting, 30 μg of protein lysate was separated by SDS-PAGE using standard procedures. The percentage of the SDS-PAGE gels was adjusted according to the molecular weight of the protein of interest. Following separation by gel electrophoresis, lysates were electroblotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore) using a wet transfer tank in transfer buffer (195

** http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf

mM glycine, 25 mM Tris, 20% (v/v) methanol). Membranes were then blocked for at least 1h with 5% (w/v) low fat milk in Tris buffered saline (50 mM Tris pH 8, 150 mM NaCl) with 0.05% Tween-20 (TBS-T). Membranes were incubated for 1 hour in a primary antibody solution diluted in TBS-T. Membranes were washed in TBS-T (three times 10 min) and then probed with the appropriate horseradish peroxidase (HRP)-coupled secondary antibody (Amersham Biosciences). After further washes in TBS-T, protein bands were visualised using the enhanced chemiluminescence (ECL) method (PerkinElmer Life Sciences, Inc.). All antibodies used in the present study are shown in Table 2.2.

Antibody	Company	Mon/polyclonal	Dilution
Akt	Cell Signalling Technology	rabbit pAb	1:2000
Akt (anti-phospho Ser 473)	Cell Signalling Technology	rabbit pAb	1:2000
Annexin II	BD Transduction Labs	mouse mAb	1:2000
Cdk4	Santa Cruz	rabbit pAb	1:5000
DUSP1 (MKP1)	Santa Cruz	rabbit pAb	1:1000
EGFR	Santa Cruz	rabbit pAb	1:2000
ErbB-2	Santa Cruz	rabbit pAb	1:1000
ErbB-3	Santa Cruz	rabbit pAb	1:1000
ERK1/2 activated	Promega	rabbit pAb	1:2000
ERK1/2 pan	Promega	rabbit pAb	1:2000
FOXM1 (MPP2)	Santa Cruz	rabbit pAb	1:1000
GFP	Clontech	mouse mAb	1:5000
IGFBP3	Diagnostic Systems Labs	goat pAb	1:5000
ISGF3γ (p48)	Santa Cruz	rabbit pAb	1:1000
ISGF3γ (p48)	BD Transduction Labs	mouse mAb	1:2000
Mn SOD	Upstate	rabbit pAb	1:2000
Myc	Santa Cruz	mouse mAb	1:500
P21	Gift from Eric Lam (Imperial College, London)	Hybridoma supernatant	1:5
P27	BD Transduction Labs	mouse mAb	1:1000
Peroxiredoxin 1 (PRDX1)	Lab Frontier	rabbit pAb	1:2000
Prohibitin (PHB)	Lab Vision	mouse mAb	1:5000
S100A6	Sigma	mouse mAb	1:1000
S100P	BD Transduction Labs	mouse mAb	1:1000
Stat1 (anti-phospho Tyr701)	Cell Signalling Technology	rabbit pAb	1:5000
Stat1	Santa Cruz	rabbit pAb	1:3000
Stat2	Santa Cruz	rabbit pAb	1:3000
Stat3	Cell Signalling Technology	rabbit pAb	1:1000
Stat3 (anti-phospho Tyr705)	Cell Signalling Technology	rabbit pAb	1:2000

Table 2.2: List of antibodies used for western blotting in the present study.

2.3.3 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

For 2D-PAGE experiments cells were starved and stimulated as desired and then lysed in 1 mL 2D-PAGE lysis buffer (4% (w/v) CHAPS, 2 M thiourea, 8 M Urea, 65 mM DTT, 10 mM Tris pH 8.3) containing protease and phosphatase inhibitors as above. Samples were scraped into eppendorf tubes and homogenised by passing through a 25-gauge needle six times. Cell lysates were centrifuged at 14,000 rpm to clear cell debris for 10 min and protein concentrations were determined using the Bradford assay. 150 μg of total cell lysate was taken for 2D-PAGE and the final volume was adjusted to 350 μL with 2D-PAGE lysis buffer and containing 2% (v/v) of a 50% (v/v) ampholine/Pharmalyte pH 3-10 mixture (Amersham Biosciences), 65mM DTT and bromophenol blue. Samples were applied to immobilised 18 cm non-linear pH 3-10 immobilized pH-gradient (IPG) strips (Amersham Biosciences) by over-night gel rehydratation at room temperature.

Isoelectrofocusing was performed on a Multiphor II apparatus (Amersham Biosciences) at 20°C for a total of 80 kVhrs. IPG strips were then equilibrated in equilibration buffer (EB) (50 mM Tri-HCl pH 6.8, 6 M Urea, 30% (v/v) glycerol, 1% SDS) with gentle rocking. The equilibration was carried out for 15 min in EB containing 65 mM DTT and then for a further 15 min in EB containing 240 mM iodoacetamide (IA) to alkylate reduced thiol groups. Equilibrated IPG-strips were then transferred onto 18 cm x 20 cm x 1.5 mm thick 12% acrylamide/0.8% bis-acrylamide gels. IPG strips were overlaid with 0.5 % (w/v) of low-melting point agarose in running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS) containing bromophenol blue. Gels were run in Protean II tanks (BioRad Laboratories) at 10 mA per gel at 10°C until the dye front had run off the bottom. Separated proteins were then blotted onto a PVDF membrane and probed with specific antibodies as described above.

2.3.4 Media-swap experiments

In order to study potential autocrine/paracrine signalling, media-swap experiments were carried out. Freshly plated HB4a and C3.6 cells were serum-starved for 24 hours and stimulated with FCS for 4 and 24 hours or with EGF, HRG, IFNβ or INFγ for 4 hours. Growth media was then removed and cells were washed extensively with PBS. Fresh, serum-free media was added to these cells and left for a

further four hours to allow secretion of molecules into the new media. This conditioned media (CM) was then added to a separate cultures of freshly plated, serum-starved cells for 15 min or 24 hours. For inhibition of ErbB activity, AG1478 was added to cells 1 hour prior to addition of CM. Cells were then lysed and subjected to western blotting.

2.3.5 *Immunoprecipitation*

Cells were lysed in NP40 lysis buffer as described above. 500 μg of total cellular lysate was used for immunoprecipitations (IP). After spinning the samples for 10 min, the cleared supernatant was collected and incubated with 2 μg of primary antibody and protein-A coupled to sepharose beads for 2 hrs at 4°C on a rotating wheel. A small amount of supernatant was collected prior to incubation with beads and antibody to allow the measurement of protein expression in total cell lysate in the same samples used for IP. Immuno-complexes were washed 5 times in NP40 lysis buffer, re-suspended in 60 μl sample buffer and boiled for 5 min at 100°C prior to separation by SDS-PAGE. Immunoprecipitates were then analysed by western blotting with the appropriate antibodies.

2.3.6 *Proliferation assay*

Cellular proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT is a yellow tetrazolium salt that is reduced by viable cells into purple formazan crystals, which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of cells is directly proportional to the level of the formazan product created, and the colour can be quantified using a simple colorimetric assay.

MTT assays were performed as follows. Cells were counted using a hemocytometer and 1000 cells per well were plated into 96-well plates. They were allowed to grow for 24 hours and then were either serum-starved in 100 μL of starving media for a further 24 hours or stimulated without starvation. Media was aspirated from wells and cells were washed once in PBS. 50 μL of 1 mg/mL MTT,

diluted in PBS and filter-sterilized, was added to each well, and the plate was incubated at 37°C for 5 hours. 100 µL of dimethyl sulfoxide (DMSO) were then added to each well to solubilise formazan crystals. Plates were placed in a shaking platform for 10 minutes at room temperature and then the absorbance was measured at 540 nm using a standard microtitre plate spectrophotometer. The HB4a un-stimulated samples were used as the control sample, and the proliferation of all other samples were represented as a percentage of this control.

2.3.7 *Immunofluorescence*

Sub-cellular localisation of different proteins was investigated by immunofluorescence. Cells were counted and seeded into 12 well plates ($2x10^5$ cell per well) containing sterile cover slips and allowed to grow to 80% confluency (generally 24 hours after plating). Cells were serum starved for 24 hours using starvation media and treated with growth factors or interferon as desired. Cells were washed in ice cold PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. They were then permeabilised with 0.2% (v/v) Triton-X100 in TBS for 5 min, washed twice in TBS and blocked in 3% BSA for 30 min at room temperature. Cells were then incubated with primary antibodies for 30 min at 37°C. Primary antibodies were used at a dilution of 1:200 in TBS. Double staining was carried out for all immunofluorescence experiments. Thus, a second incubation with a primary antibody from a distinct species was carried out under identical conditions. Goat anti-mouse antibodies conjugated to tetramethylrhodamine isothiocyanate (TRITC) and goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) (Jackson Immunoresearch Laboratories Inc.) were used for the detection of primary antibodies. Secondary antibodies were used at a 1:500 dilution. Coverslips were then mounted on a glass microscope slide with DAKO fluorescent mounting medium (DAKO Corporation). Images were collected with a cooled CCD camera (Photonic Science) mounted over an Axiophot microscope fitted with an X40 oil immersion objective (Zeiss, UK). All images collected with the CCD camera were taken under the same magnification and exposure time.

2.3.8 Transfection experiments

The ErbB2 cDNA used in these experiments was cloned into the pCDNA3 expression vector, in which the gene is expressed from the CMV (cytomegalovirus) promoter. ErbB2-pCDNA3 and the empty vector (pCDNA3) used as a control were kindly provided by Dr. Colleen S. Crovello, Harvard Medical School, Boston, USA. The EGFP-C2 DNA was a kind gift from Dr. Peter Downs, University of Dundee, UK, and was used to co-transfect cells in order to determine transfection efficiency. Competent *E.coli* were transformed with plasmids using standard procedures and plasmid DNA isolated using the QIAGEN Maxiprep kit (QIAGEN) following the manufacturer's instructions. Plasmid concentration was determined in a spectrophotometer at 260 nm, where 1 OD unit=50 μg/mL. The OD_{260}/OD_{280} ratio, which is an indication of nucleic acid purity and should be as close to 1.8 as possible for pure DNA, was determined for all DNA preps used in this study and were as follows: 1.72 (ErbB2-pCDNA3), 1.76 (empty vector) and 1.77 (EGFP-C2).

Transfection experiments were carried out using the FuGENE 6 transfection reagent (Roche Applied Science). Cells were seeded 24 hours prior to transfection into 6-well plates and were normally ~80% confluent on the day of transfection. FuGENE was diluted in OPTIMEM medium (Gibco-Invitrogen Corp.) to a total of 100 μL/well. 2 μg of ErbB2 and EGFP or empty vector and EGFP DNA were added to the FuGENE/OPTIMEM mixture and incubated at room temperature for 30 minutes. Two FuGENE (μL):DNA (μg) ratios were used in these experiments, 6:1 and 3:1, and the volume of FuGENE reagent was adjusted accordingly. The DNA/FuGENE complex mixture was then added dropwise to wells containing complete growth media. Cells were incubated for 24 hours at 37°C and assayed for transfection efficiency using a fluorescence microscope. Cells were then treated as desired, lysed and subjected to western blotting as described above.

Chapter 3: IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES BY MICROARRAY TECHNOLOGY

3.1 Chapter Introduction

ErbB2 is overexpressed in 25-30% of all breast cancers and its overexpression is associated with poor prognosis (Ross & Fletcher 1999). Although its role in cancer is well documented, the impact of ErbB-2 overexpression on the molecular mechanisms involved in the development of cancer remains unclear. Microarray technology is a powerful tool for parallel analysis of thousands of genes simultaneously and its usefulness in the study of cancer has been well established (see Chapter 1). However, few studies report the use of microarrays in the study of ligand-induced signalling events in a context relevant to breast cancer, such as ErbB2 overexpression. This issue was addressed in this study, where a number of microarray experiments were performed to evaluate the role of ErbB2 in cellular signalling and transformation. These experiments were carried out using a relevant model cell system consisting of a parental breast luminal epithelial cell line HB4a, and an ErbB-2 overexpressing derivative, C3.6 (Harris *et al.* 1999). This Chapter describes the comparison between these two cell lines by microarray technology and the analysis of the downstream gene expression changes associated with ErbB2 overexpression and ErbB receptor signalling through the use of specific ligands. Such analysis may help identify targets of ErbB2 overexpression and shed light on the signalling events downstream of ErbB receptors in both the normal and transformed cellular states.

3.2 Characterization of Cell Lines Used in this Study

The study of the effects of ErbB2 overexpression and growth factor signalling on gene expression was carried out by microarray analysis of the human mammary luminal epithelial cell (HMLEC) lines HB4a and C3.6. Previous studies have shown that the ErbB2 overexpressing clone (C3.6) displays growth factor-independent signalling, has an increased responsiveness to mitogens and displays a higher rate of proliferation compared with HB4a cells, largely due to elevated ERK1/2 signalling

(Harris *et al.* 1999,Timms *et al.* 2002). The higher proliferative rate is characterized by increased levels of the cell cycle regulators cyclin D1 and cyclin E and a lower level of the cdk inhibitor p27, resulting in a more rapid G1 phase transition and early cell cycle entry after acute stimulation with mitogens (Timms *et al.* 2002). The levels of ErbB2 expressed at the cell surface were previously measured by FACS analysis and compared to well defined breast cancer cell lines overexpressing ErbB2 (BT474 and SKBr3) (Harris *et al.* 1999). From these results, the level of overexpression of ErbB2 in the C3.6 cells was estimated to be approximately 10-fold higher than that of the parental cell line, levels which are similar to that seen in many human breast tumours.

The total cellular levels of ErbB2 were estimated by western blotting in the HB4a and C3.6 cell lines (Figure 3.1-a). This data showed that the levels of ErbB2 were similar to the levels estimated previously by FACS analysis (Harris *et al.* 1999). The level of other ErbB family members was also examined, and this confirmed previous findings that C3.6 cells express lower levels of EGFR but higher levels of ErbB3, suggesting that ErbB2 overexpression could modulate the expression of other ErbB receptors. ErbB4 was not detected in either cell line (data not shown). These changes are likely to influence the heterodimerisation pattern of the ErbB family and hence the specificity of downstream signalling in the two cell lines. Phase contrast microscopy was also used to obtain cell images and determined cellular morphological characteristics. The C3.6 cells were found to be more elongated, refractory and tightly packed than the HB4a cells (Figure 3.1-b). These data suggest that ErbB-2 overexpression results in altered cellular phenotype, manifested as a change in cellular morphology and possibly in altered cell-cell interactions and adhesion. Indeed, previous work has shown that the C3.6 cells are less adherent and able to proliferate as colonies in soft agar (Harris *et al.* 1999,White *et al.* 2004).

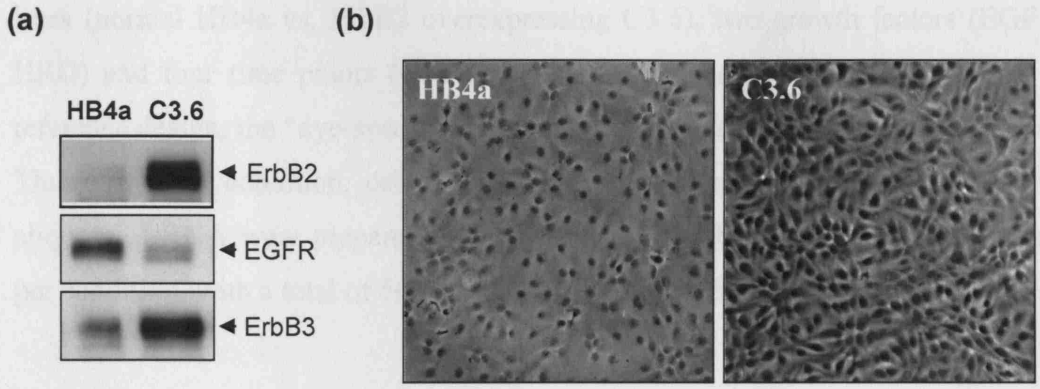

Figure 3.1: Characterization of HB4a and C3.6 cell lines. (a) ErbB receptor levels were determined for each cell line by Western blotting using specific antibodies for ErbB2, EGFR, ErbB3 and ErbB4. ErbB4 was not detected these cells (data not shown). **(b)** Phase-contrast microscopy was used to check for characteristic morphological differences between HB4a and C3.6 cells.

3.3 Microarray Experimental Design

The aim of these experiments was to investigate the effects of ErbB2 overexpression on gene expression as well as the effect of time-dependent stimulation of HB4a and C3.6 cell lines with EGF and HRG, which would activate EGFR and ErbB3 signalling, respectively. Since a large number of variables needed to be analysed and cross-compared, the reference design was used in the present study. The work reported by White *et al* (White *et al.* 2004), which was also carried out in our lab, provides a good basis for choosing this experimental design. Using the same cell system, the authors used microarrays to study the effect of ErbB2 overexpression as well as time-dependent changes in gene expression following HRG stimulation. For each time point, HB4a and C3.6 cells were co-hybridized onto the same slide and therefore the relative gene expression levels in C3.6 cells were directly compared to HB4a, and determined by the ratio (C3.6/HB4a). Whereas they observed time dependent changes in the expression of a number of genes, these results were difficult to interpret as it was not possible to determine the direction of the gene expression changes over time, only between cell lines at a particular time point. Thus, for a microarray experiment of such complex nature, the reference design is more desirable as it allows comparison between all experimental conditions

equally. In this study, a total of 14 conditions were analysed. They were: two cell lines (normal HB4a vs. ErbB2 overexpressing C3.6), two growth factors (EGF vs. HRG) and four time points (serum-starved, 4h, 18h, and 24h). In addition to the reference design, the "dye-swap" strategy was adopted to minimize dye-specific bias. Thus, for each condition, cell lines were grown in two separate plates and two aliquots of RNA were prepared from each plate, thereby generating four replicates per condition, with a total of 56 hybridizations (Figure 3.2).

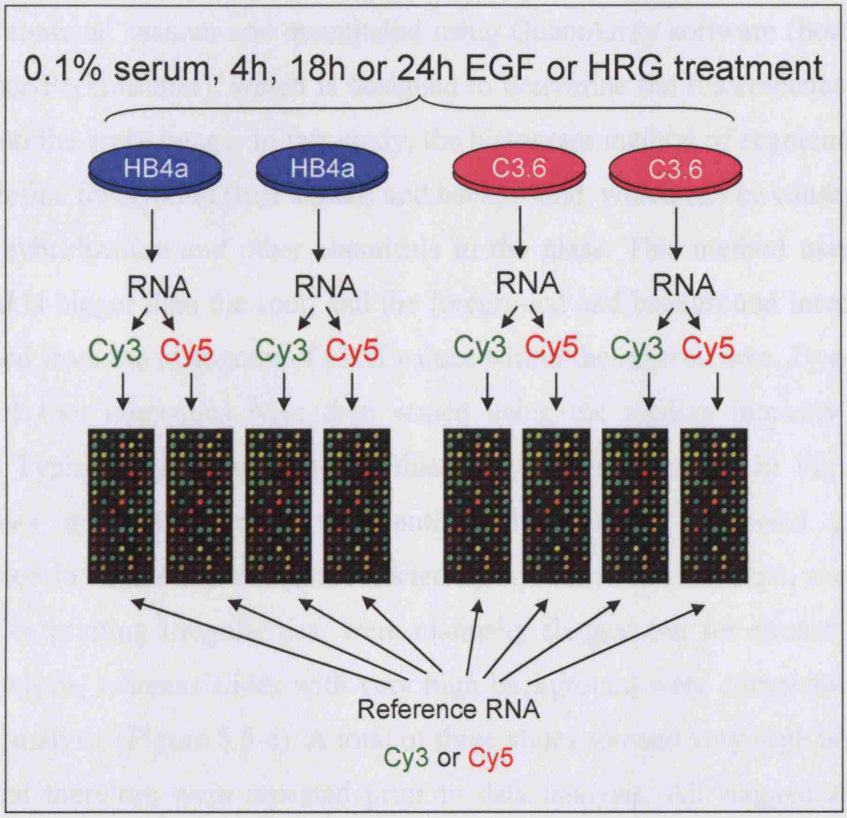

Figure 3.2: Microarray experimental design used in this study. HB4a and C3.6 cells were stimulated with EGF or HRG for the indicated time points. Two plates were prepared for each experimental condition, from which two RNA samples were prepared and used for hybridization using the "dye-swap" strategy. All samples were co-hybridized to a common reference sample, which consisted of pooled RNA from BT474 breast carcinoma cell line and two grade III invasive ductal breast carcinomas (kindly provided by Alan Mackay, Institute for Cancer Research). Thus, four replicates per condition were carried out, resulting in a total of 56 hybridization experiments.

3.4 Microarray Data Analysis

3.4.1 Image acquisition, spot quantitation and data normalization

As discussed in Chapter 1, there are various data analysis strategies that can be used for microarray data. It is important to carry out the necessary steps to eliminate systematic variations and to identify genes of interest. Thus, before analysing the data collected from the hybridizations, images were quality controlled and raw data normalized to enable a more accurate estimate of true differential gene expression. Following hybridization, each image was scanned using ScanArray 4000XL confocal scanner and quantitated using QuantArray software (both Packard BioScience/PerkinElmer), which is designed to determine the fluorescence intensity of spots on the array image. In this study, the histogram method of segmentation was used to define foreground (true signal) and background, which can be caused by non-specific hybridization and other chemicals in the glass. This method uses a target mask that is bigger than the spot, and the foreground and background intensities are determined from the histogram of pixel values within the masked area. Dye-emission signals of spot intensities were then scaled using the median intensity for each channel. Typical images acquired in this study are represented in Figure 3.3-a. Differences in spot intensity representing differentially expressed genes are exemplified in Figure 3.3-b. Spots affected by hybridization artefacts, such as dust particles or printing irregularities, were manually flagged out for exclusion during image analysis, whereas slides with very high background were completely ignored from the analysis (Figure 3.3-c). A total of three slides showed very high background levels, and therefore were repeated prior to data analysis. All flagged spots were recorded as missing. Thus, great care was taken during the image processing step so that downstream data analysis would not be compromised by poor quality images and spot quantitation.

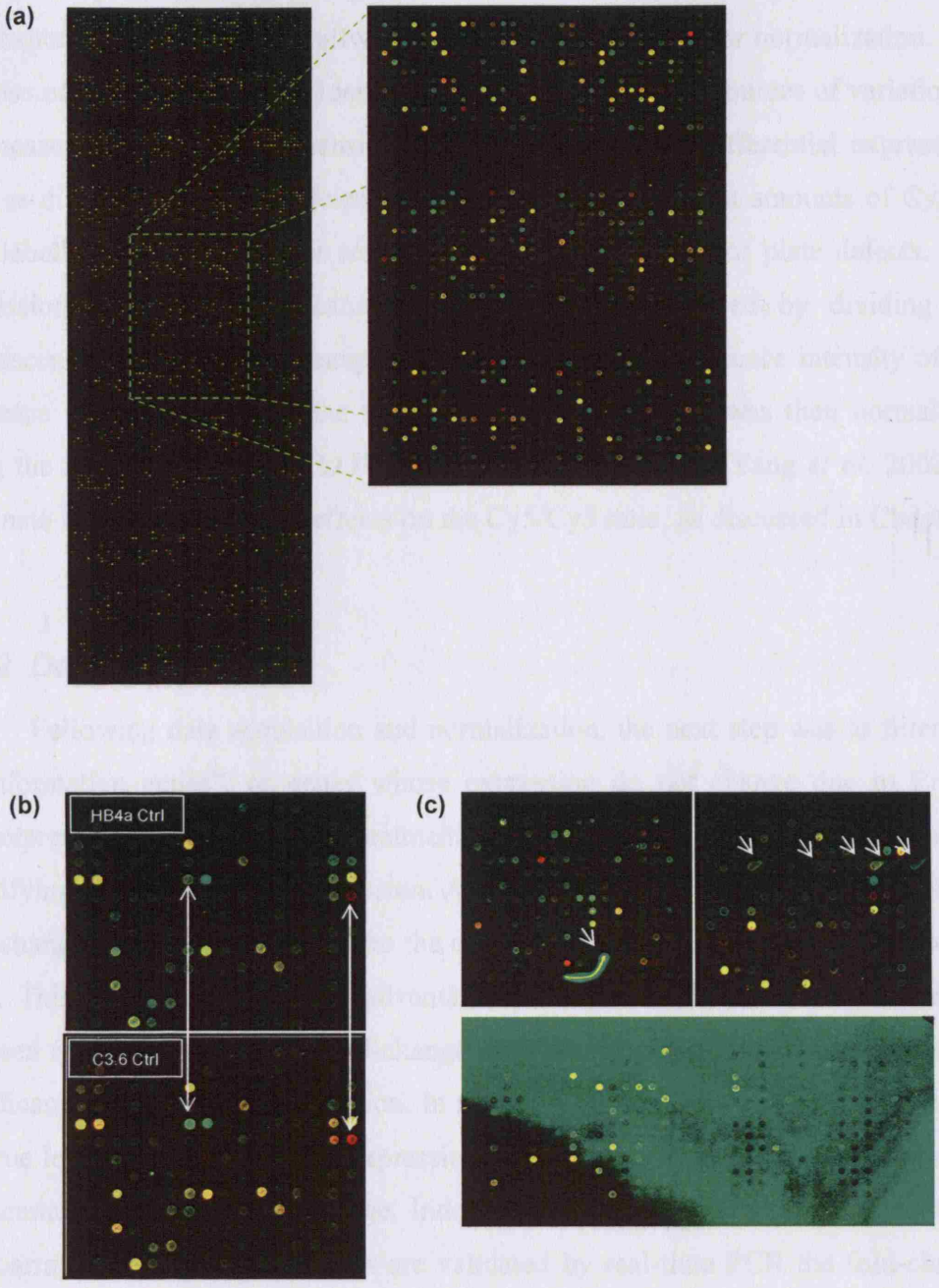

Figure 3.3: Microarray images generated in this study. (a) Typical whole-slide image. After scanning the two channels with different excitation emissions for each Cy dye, the acquired images were merged and pseudo-coloured. Pixels were then quantitated to identify genes differentially expressed between samples. **(b)** Examples of differential gene expression between normal HB4a cells and ErbB2-overexpressing C3.6 cells. The examples shown are for KRT15 and KRT13, both of which were found to be significantly upregulated in C3.6 cells following statistical data analysis. **(c)** Spots affected by hybridization artefacts such as dust particles or irregular shapes (upper panel, white arrows) were excluded from downstream data analysis. Slides with very high background levels (lower panel) were completely excluded from analysis and the hybridization was repeated.

Background subtracted signal intensities of the Cy3 and Cy5 channels were then exported to GeneSpring software v6.1 (Silicon Genetics) for normalization. The purpose of normalization is to identify and remove systematic sources of variation in the measured fluorescence intensities which are not due to differential expression, such as different labelling efficiencies of the Cy dyes, different amounts of Cy3 or Cy5 labelled mRNA, different scanning parameters, print-tip or plate defects. The expression level for each feature on the array was derived by dividing the fluorescence intensity of the sample of interest by the fluorescence intensity of the reference sample in each of the four replicates. The dataset was then normalized using the intensity-dependent LOWESS regression technique (Yang *et al.* 2002) to eliminate intensity-dependent effects on the Cy5/Cy3 ratio, as discussed in Chapter 1.

3.4.2 Data filtering

Following data acquisition and normalization, the next step was to filter out "uninformative genes", or genes whose expression do not change due to ErbB2 overexpression or growth factor treatment. There are various strategies available for identifying changes in gene expression. A commonly used strategy is to look at the fold-change of the sample relative to the control, with a cut-off of 2-fold often being used. This technique has the disadvantage that it does not account for variation between replicates and a high fold-change may not necessarily reflect a statistically significant change in gene expression. In addition, fold-changes are not indicative of the true levels of over- or under-expression, as microarrays are not sensitive enough to accurately quantitate fold-change. Indeed, as will be discussed Chapter 4, when microarray gene expression results are validated by real-time PCR the fold-change for the same gene is normally larger in the PCR experiment, reflecting the higher sensitivity of this technique. Therefore, fold-change is not a good method to infer significance as genes with a low, but consistent, fold-change may well represent true differentially expressed genes.

In this study, significant changes in gene expression were identified using SAM, a statistical methodology useful for screening significant gene expressions from microarray data (Tusher *et al.* 2001). The SAM method is explained in the Materials & Methods Chapter. This analysis identified 775 genes whose expression levels differ significantly in one or more conditions, i.e. genes which are responsive

to EGF or HRG at one or more time points, genes differentially regulated by ErbB2 overexpression, or both.

3.4.3 Data analysis

One of the principal aims of this study was to identify patterns of gene expression that would indicate biologically significant groups of genes whose differential expression contribute to ErbB2-dependent breast cancer development. There was also a great interest in determining genes co-regulated by growth factors in order to compare and contrast signalling events induced by EGF and HRG. Clustering techniques allow rapid visualisation of differentially expressed genes and therefore are a good starting point in microarray data mining. As the intensity values were initially represented by the ratio sample/reference, it was necessary to determine an additional ratio prior to clustering to obtain expression levels relative to a control sample depending on the variable being studied. Thus, the ratios of the normalized values were calculated in two ways: (i) the 'ErbB2 ratio' (C3.6/HB4a) represents the relative gene expression in C3.6 cells versus HB4a at each time point thereby identifying genes whose expression are affected by ErbB2 overexpression, and (ii) the 'EGF ratio' or 'HRG ratio' (T*/T0h) represents the relative gene expression in a cell line in relation to the untreated control and therefore measures how genes respond to each ligand in HB4a and C3.6 cells separately (Figure 3.4). These values were then $\log_2$-transformed before carrying out clustering analysis.

Figure 3.4: Two different types of ratios used for clustering analysis. The ErbB2 ratio was used to determine ErbB2-dependent changes in gene expression, whereas the GF ratio was used to estimate growth factor-dependent changes in gene expression in a time-dependent fashion.

Initially, Principal Component Analysis (PCA) was performed. This technique reduces the dimensionality of the data and therefore facilitates the identification of distinct groups of genes with specific gene expression profiles. PCA was applied to the 'ErbB2 ratio' for each growth factor separately to try and identify groups of genes with different growth factor responsiveness between the two cell lines. This analysis yielded no readily apparent gene clusters and it was not possible to identify groups of genes whose expression differed more significantly in a given condition (data not shown). Hierarchical clustering was then carried out on all 775 genes identified by SAM on both 'ErbB2 ratio' and 'GF ratio' samples (Figure 3.5). Although no striking functional expression clusters were apparent at first glance, a few points were of interest. Firstly, genes whose expression levels are changing more markedly in the ErbB2 ratio samples show similar levels of up- or downregulation with both EGF and HRG (light green and pink bands, Figure 3.5). This suggests that these genes are directly regulated by ErbB2 overexpression and therefore may represent important markers of ErbB2-related breast cancers. Secondly, a group of genes that was downregulated in the ErbB2 ratio in growth factor simulated samples actually get upregulated in response to growth factor treatment (light blue band). This likely reflects the difference in responsiveness to growth factors between the two cell lines, and highlights the importance of the reference sample design for microarray experiments of such complex nature. In addition, at the top of the cluster there were two features which did not cluster with the remaining 773 genes. These were both found to be IGFBP3, a regulator of IGF-dependent and independent cellular growth, proliferation, differentiation and survival (Ricort & Binoux 2004). IGFBP3 was highly downregulated in C3.6 cells, and its expression was found to be significantly downregulated following EGF stimulation (Figure 3.6). The fact that these clones did not cluster with any other genes stems from the high level of down-regulation in response to growth factor. No other gene identified in this analysis showed such a marked response to growth factor and such a large difference in expression level between the cell lines. A small number of genes were however downregulated in response to growth factor alone, particularly HRG (dark green band). Finally, a group of transiently-induced genes (up at 4 hours) was identified in all GF treatments, except the HRG-treated HB4a samples (red band). These differences in growth factor responsiveness between the cell lines is almost certainly

due to the fact that HB4a cells express lower levels of ErbB3 and ErbB2 (Figure 3.1 and (Timms *et al.* 2002)), reflecting this poorer response to HRG.

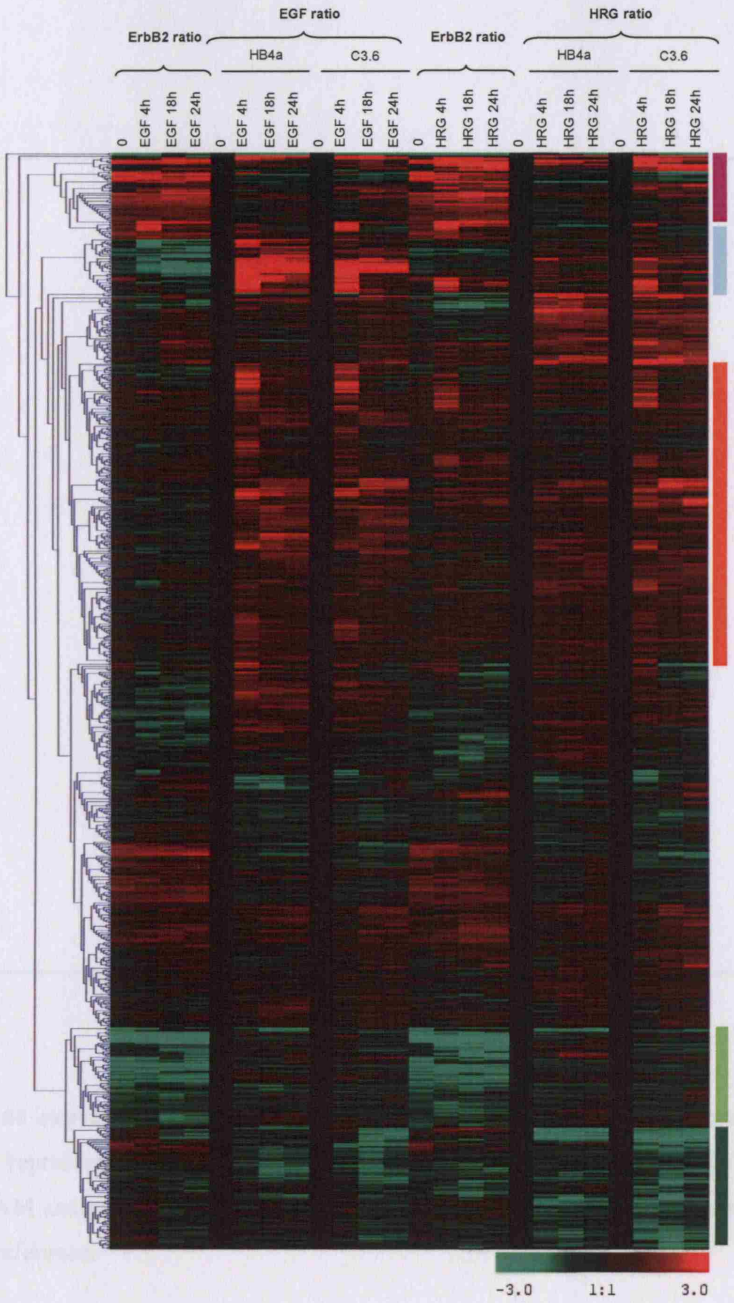

Figure 3.5: Hierarchical clustering of 775 genes identified to be changing in one or more conditions using SAM. Fold changes were calculated using the GF and ErbB2 ratios described in Figure 1.5. Gene expression levels were then coloured according to the level of over- (red) or under- (green) expression across all experimental samples. Average-linkage clustering was performed using Euclidean distance measurement.

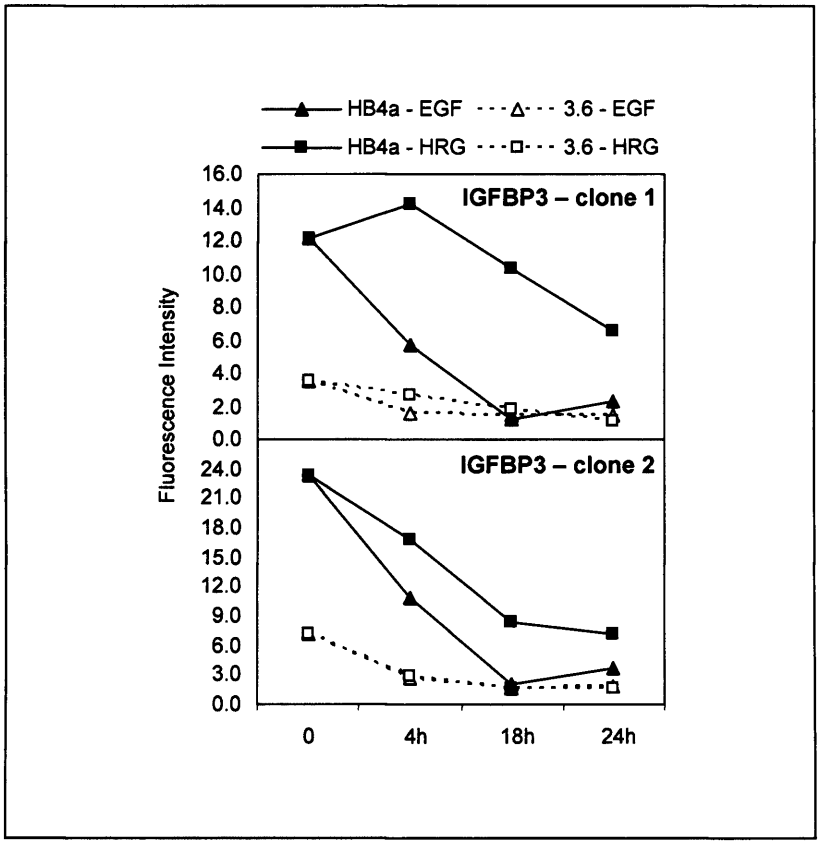

Figure 3.6: Gene expression pattern of the IGFBP3 gene. Graphical representation of the two IGFBP3 clones represented on the array and found to be significantly changing in one or more conditions by SAM analysis. The values on the y-axis represent the normalized fluorescence intensity ratio of sample/reference.

Since pair-wise comparisons were performed, this only allows the identification of differentially expressed genes due to differences in ErbB2 levels or those only regulated by EGF or HRG. However, by using a Venn diagram, it was possible to analyse the overlap between these gene groups and identify those which are responsive to more than one condition (Figure 3.7). Gene lists for each area of the Venn diagram were imported into excel to enable a rapid and easy way to look up genes present in each group. This table can be found in Appendix 1, where all 775 genes are grouped and listed. An important factor that is readily noticeable is the presence of the same gene in different lists. A gene can be represented on the array more than once, where a different sequence of the same gene is spotted. For example, the gene HIBCH, which is involved in cellular metabolism, is present in the "ErbB2 only" list as well as in the "HRG & ErbB2" list. Indeed, when the profile of HIBCH was compared, the expression levels differed between the two represented clones, as did copine 3 (CPNE3) (Figure 3.8-a). Results like this are difficult to interpret and require further validation using other methods to determine the true differences in expression level. On the other hand, genes represented more than once that are present on the same list allow an extra level of confidence in the results, and indeed this was the case with many of the genes identified in this analysis (Figure 3.8-b).

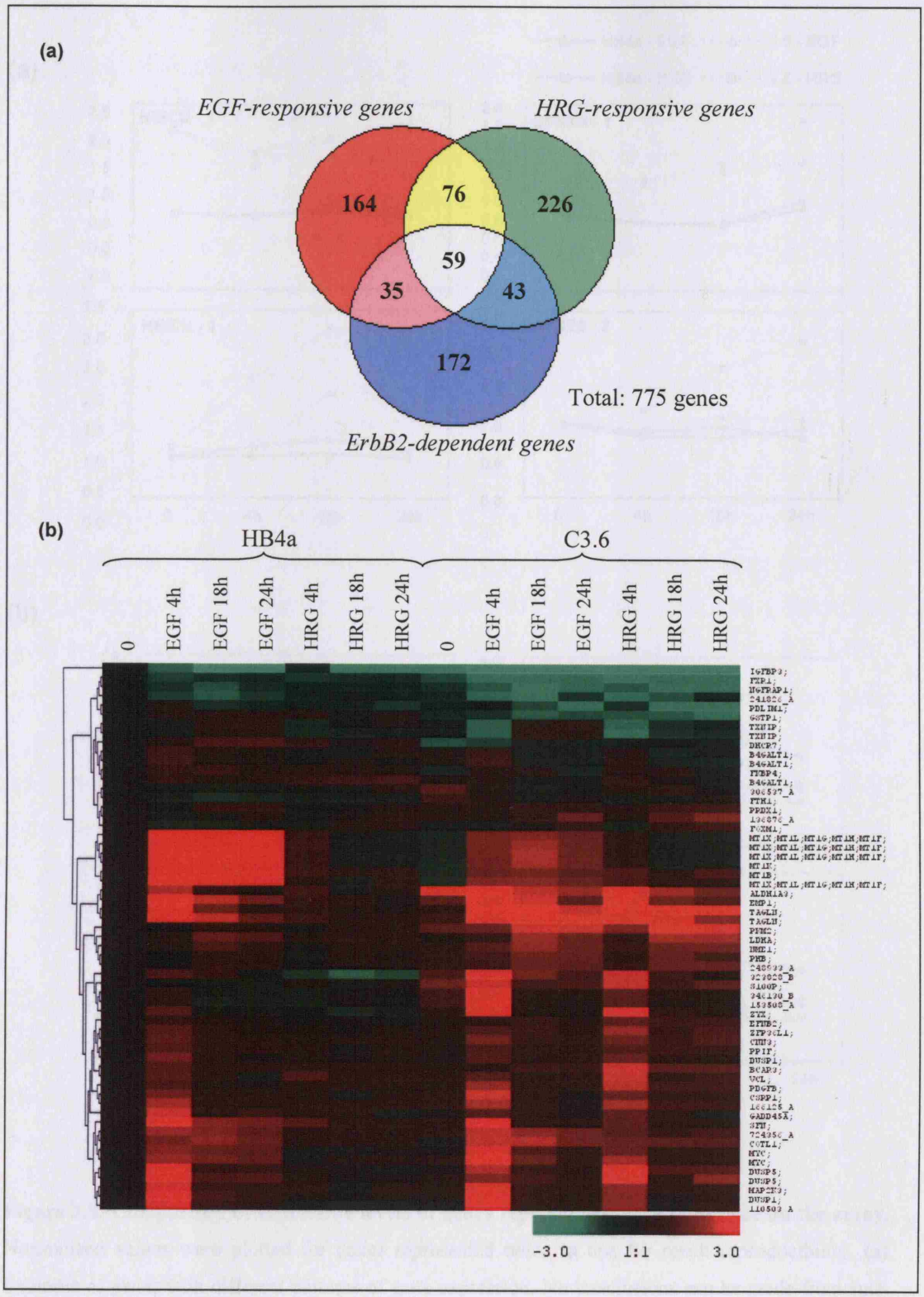

Figure 3.7: Separation of the 775 genes according to ErbB2 or growth factor dependency. (a) Venn diagram of genes responsive to EGF or HRG and genes whose expression are altered by ErbB2 overexpression. **(b)** Hierarchical clustering of the 59 genes changing in response to EGF, HRG and ErbB2.

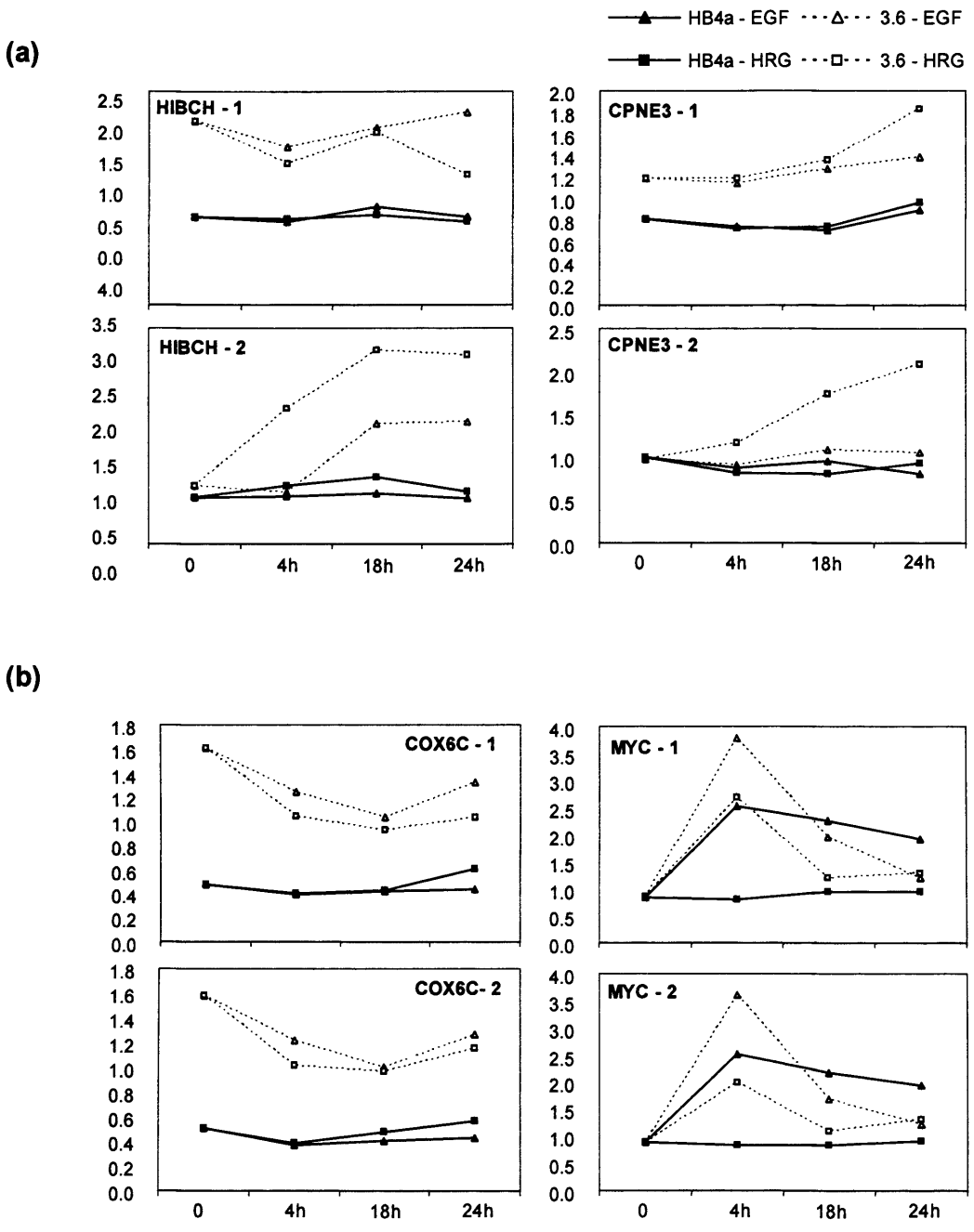

Figure 3.8: Comparison of expression levels of genes represented more than once on the array. Normalized values were plotted for genes represented twice to test for result reproducibility. **(a)** Example of genes with different patterns of gene expression. No conclusions can be made from such measurements unless results are validated using alternative methods for measuring gene or protein expression. **(b)** Genes with highly similar expression patterns when represented more than once. These patterns are more likely to represent the true levels of expression and increase the confidence in the results.

Of the 775 differentially expressed genes, 59 were found to be changing in response to all conditions, i.e. EGF, HRG, and ErbB2 overexpression at one or more time points. As seen in Figure 3.7-b, following unsupervised hierarchical clustering of these 59 genes, IGFBP3 once again did not cluster with any of the other genes. Note that only one IGFBP3 clone was present on this list. The second clone was also found to be downregulated in C3.6 cells and following EGF treatment, but showed no change in gene expression in response to HRG (Figure 3.6). Because of its striking pattern of gene expression and its role in regulation of proliferation and transformation (Ricort 2004), IGFPB3 was chosen as one of the genes for validation by Real-Time PCR. These results will be discussed in the next chapter.

Other genes in this list included MYC, MAP2K3 (MKK3) and the dual-specificity phosphatases DUSP1 and DUSP5, which have previously been implicated in EGF- and/or HRG-induced MAPK signalling as well as cancer progression. These findings support the legitimacy of the system used in this study. Moreover, genes that were represented twice in this list tended to cluster together (e.g. DUSP5, MYC, TXNIP, B4GALT1, TAGLN and MT family members), indicating that their expression patterns are similar and thereby reinforcing the validity of these findings. The proto-oncogene transcription factor MYC is implicated in various physiological processes, including cell growth, proliferation, loss of differentiation and apoptosis (Pelengaris & Khan 2003). Importantly, it has been shown that co-amplification of MYC and ErbB2 in breast cancers is associated with a significant reduction in patient survival (Cuny *et al.* 2000). The MYC-induced gene EMP1 (epithelial membrane protein 1, or TMP) was also upregulated in response to ErbB2, EGF and HRG (although one of the two represented clones was not significantly upregulated by HRG). EMP1 is a membrane glycoprotein, and although little is known about its function, a role in proliferation and tumorigenesis has been proposed (Ben Porath *et al.* 1998,Ben Porath *et al.* 1999). EMP1 was originally isolated from a cDNA library of a brain tumour which developed in a mouse transgenic for *c-myc* (Benvenisty *et al.* 1992). It is interesting to note that EMP1 expression is closely related to that of MYC (Figure 3.9), in agreement with its characterization as a MYC-induced gene.

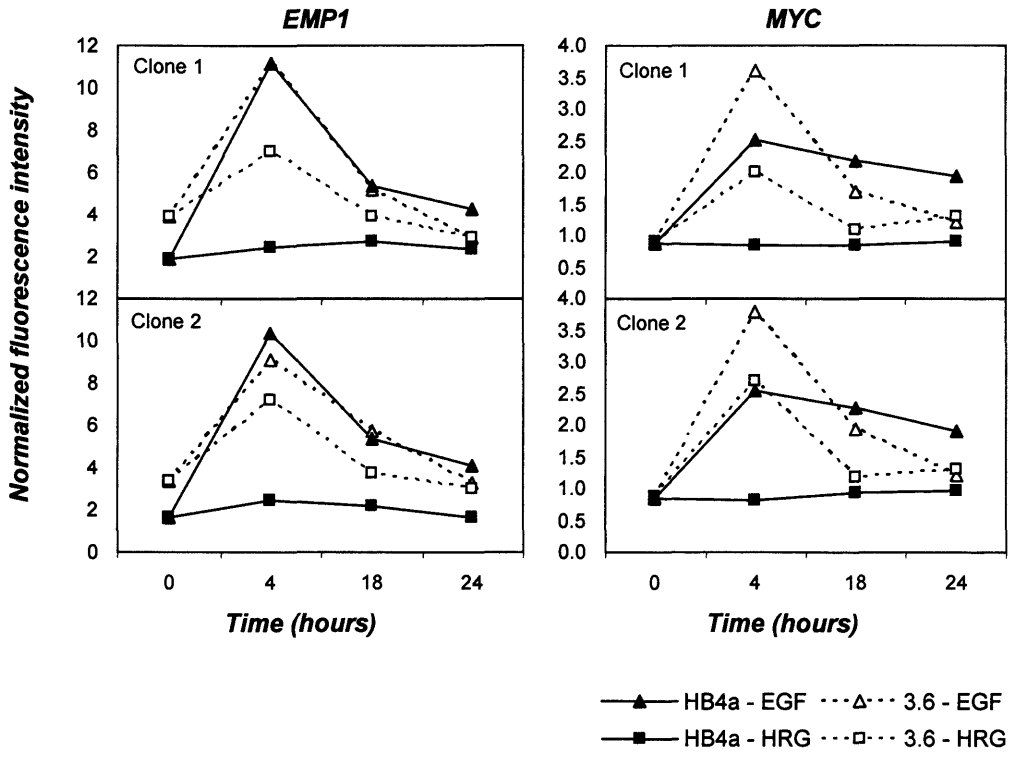

Figure 3.9: Expression patterns of EMP1 and MYC. EMP1, a gene thought to be regulated by MYC, shows a very similar expression pattern to that of MYC in response to growth factor treatment.

BCAR3 was also present in this list. This gene has been implicated in anti-oestrogen resistance in breast cancers and is believed to be involved in tyrosine kinase signalling due to the presence of a putative SH2 domain in its sequence (van Agthoven *et al.* 1998). Although BCAR3 has not been previously connected to ErbB signalling, it has been shown to be recruited to phosphorylated tyrosines (and hence hypothesized to become activated) following EGF stimulation in a proteomics study (Blagoev *et al.* 2004). Here, BCAR3 is not only upregulated by EGF, but also by HRG. This result is interesting as ErbB2 overexpression has been suggested to contribute to anti-oestrogen resistance (Shou *et al.* 2004), and therefore BCAR3 may provide a link between ErbB2 overexpression and anti-oestrogen resistance in breast cancer. A number of genes associated with actin and the cytoskeleton were also present in this gene list, including zyxin (ZYX), transgelin (TAGLN), vinculin (VCL), calponin 3 (CNN3) and elfin (PDLIM1), possibly reflecting the different cellular morphologies displayed by the HB4a and C3.6 cells. The calcium-binding

protein S100P was also present in this gene list and has been previously shown to display increased expression in breast cancer (Guerreiro, I *et al.* 2000), and was found to be overexpressed in C3.6 cells in other microarray studies (Mackay *et al.* 2003,White *et al.* 2004). However, it was represented twice on the arrays and each clone was placed in a different gene list following statistical data analysis. Although it is present in the gene list discussed here (changing in response to ErbB2 overexpression as well as in response to both growth factors), S100P is also present in the "ErbB2 only" list. Thus, while S100P expression levels are indeed higher in the ErbB2 overexpressing cells, it is not possible to determine with confidence whether its expression is affected by growth factor treatment. S100P was therefore chosen for further validation by Real-Time PCR and these results will be discussed in Chapter 4.

Various members of the MT-1 family of metallothioneins were also present in the list of genes significantly changing in all the conditions, and their expression pattern is shown in Figure 3.10. They are highly upregulated in response to both GFs, with a more potent induction of mRNA expression in EGF-treated samples. Metallothioneins are low molecular weight, cysteine-rich proteins with high affinity for heavy metals, and four major isoforms (MT-1, MT-2, MT-3 and MT-4) have been identified in mammals (Theocharis *et al.* 2004). Several lines of evidence suggest a role for MTs in cancer development, treatment resistance and prognosis. In breast cancer, MTs expression has been shown to correlate with poor patient prognosis (Goulding *et al.* 1995). It has been previously reported that 48h EGF stimulation can induce MT mRNA expression in rat hepatocytes (Moffatt *et al.* 1995). However, this is the first report on the ability of EGF, and to a lower extent HRG, to induce the expression of MT-1 isoforms in human epithelial cells. In addition, although not present in this gene list, the isoform MT-3, whose expression is thought to be restricted to brain (Palmiter *et al.* 1992), was found to be highly inducible in response to EGF in mammary HB4a cells.

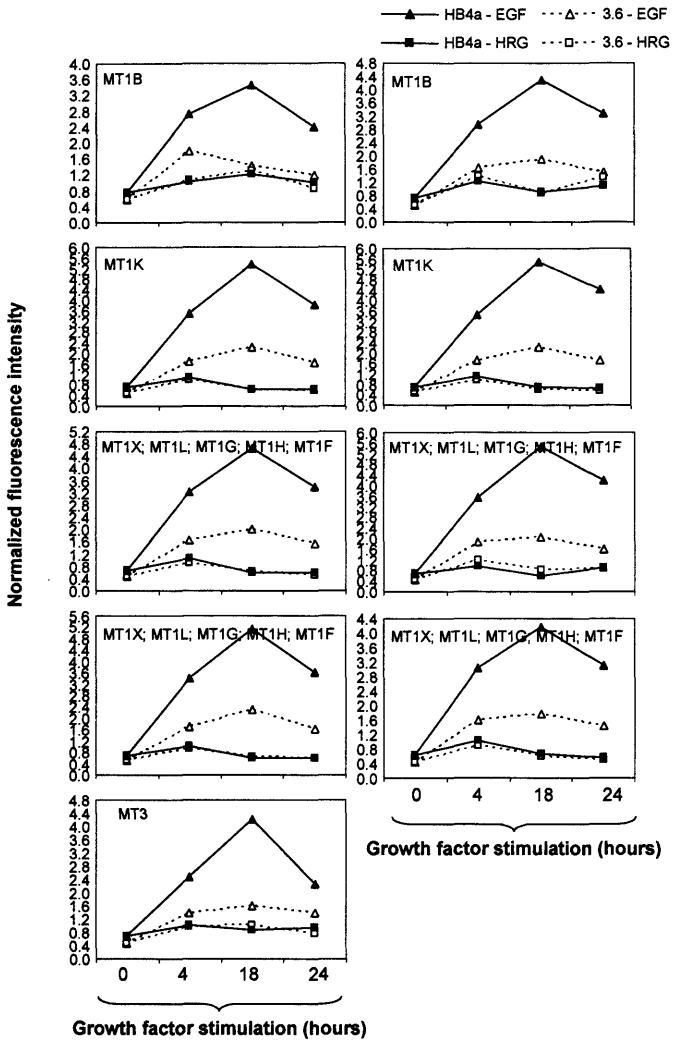

Figure 3.10: Expression levels of MT family members. Expression levels of all members of the metallothionein family are shown. All members were reproducibly highly upregulated by EGF treatment, and this induction was more marked in HB4a cells. Although some of the genes above were also found to be upregulated in response to HRG, the induction observed is significantly lower and therefore requires further experiments to test whether indeed HRG can upregulate the expression of MT family members.

3.4.4 Genes with no common name annotation

Of the 775 genes identified in this analysis as significantly changing in one or more condition, 197 features were not assigned a "common name", and are instead only labelled by a unique gene identifier (e.g. 136252_A). Because the sequences present in the microarrays were manually curated at the Sanger Centre, it is possible that they in fact represent genes that have been already annotated. Indeed, of the 197

genes that do not have a common name assigned to it, 58 have Ensembl annotations and description and 30 have Ensembl identifiers but no functional description. Ensembl is a joint project between EMBL-EBI and the Sanger Centre to develop a software system which produces and maintains automatic annotation on eukaryotic genomes (Birney *et al.* 2004), and therefore sequences present on the array that contain Ensembl description are likely to represent known genes. The annotation used in the present study was based on the Sanger Centre's data file Hver1.3.1_33. Microarray sequence annotation data files are frequently updated and the most up-to date annotation files can be downloaded from the Sanger Centre's website (http://www.sanger.ac.uk/Projects/Microarrays/informatics/datafiles.shtml).

Further annotation to clones not assigned a common gene name was obtained by searching the NCBI's GEO profile database. This database stores individual gene expression profiles assembled from the Gene Expression Omnibus (GEO) repository, and therefore facilitates searching and linking to additional information sources. Thus, it was possible to obtain further annotation from deposited gene lists of other Sanger Centre array users. By comparing the unique identifiers, which do not change between arrays, additional "common names" were identified for particular features. Sequences of greater interest (i.e. that cluster with other interesting genes) could be further verified by blasting the actual sequences spotted on the array (which can be obtained from the Sanger Centre website) to obtained sequence similarity and confirm the sequence annotation. Throughout this chapter, a small number of genes with no annotation found to cluster with other interesting genes of particular families will be discussed. These were identified on the GEO profile obtained, and further verified by sequence similarity in Blast. Web pages relevant to sequence annotation of clones present in the array are shown below in Table 3.1.

Web Page	*Web address*
GEO Profile	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo&cmd=search&term=
Sequence annotation verification protocol (Sanger Centre)	http://www.sanger.ac.uk/Projects/Microarrays/arraylab/protocol11a.pdf
Microarray Reporter Sequence information (Sanger Centre)	http://www.sanger.ac.uk/cgi-bin/microarrays/reporter_annotation?array_id=Hver&reporter_id=
BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
Ensemble	http://www.ensembl.org/

Table 3.1: Useful web pages for verification of microarray sequence annotation.

Hierarchical clustering was performed on this dataset. Four main clusters were observed (indicated by coloured dendogram branches in Figure 3.11), suggesting that such genes are indeed affected by ErbB2 overexpression and/or growth factor stimulation. Clones with no annotation were found to represent genes involved in processes previously documented in cancer, such as cell cycle and apoptosis regulation, protein phosphorylation (DOC-1R, TRAF2 and HLCDGP1, respectively), cellular response to oxidative stress (GSTO1) as well as cytoskeleton-related genes (myosin regulatory light chain 2, or MYL2). It is also worth noting that some of the un-annotated genes were found to be different clones of genes that were annotated and displayed altered expression. For instance, the prohibitin (PHB) gene, which was upregulated in C3.6 cells and following EGF and HRG stimulation, was also found to correspond to the clone 248933_A. This clone showed an almost identical pattern of expression, and clustered together with the PHB gene (see Figure 3.7), further validating the expression change of PHB. However, a number of clones were indeed of unknown function. Thus, although the characterization of all the genes with no annotation is well beyond the scope of this project, these results suggest that there may be novel genes involved in ErbB signalling pathway that have potentially important implications in the development of breast cancer. Further studies are required in order to characterize the unknown genes identified here.

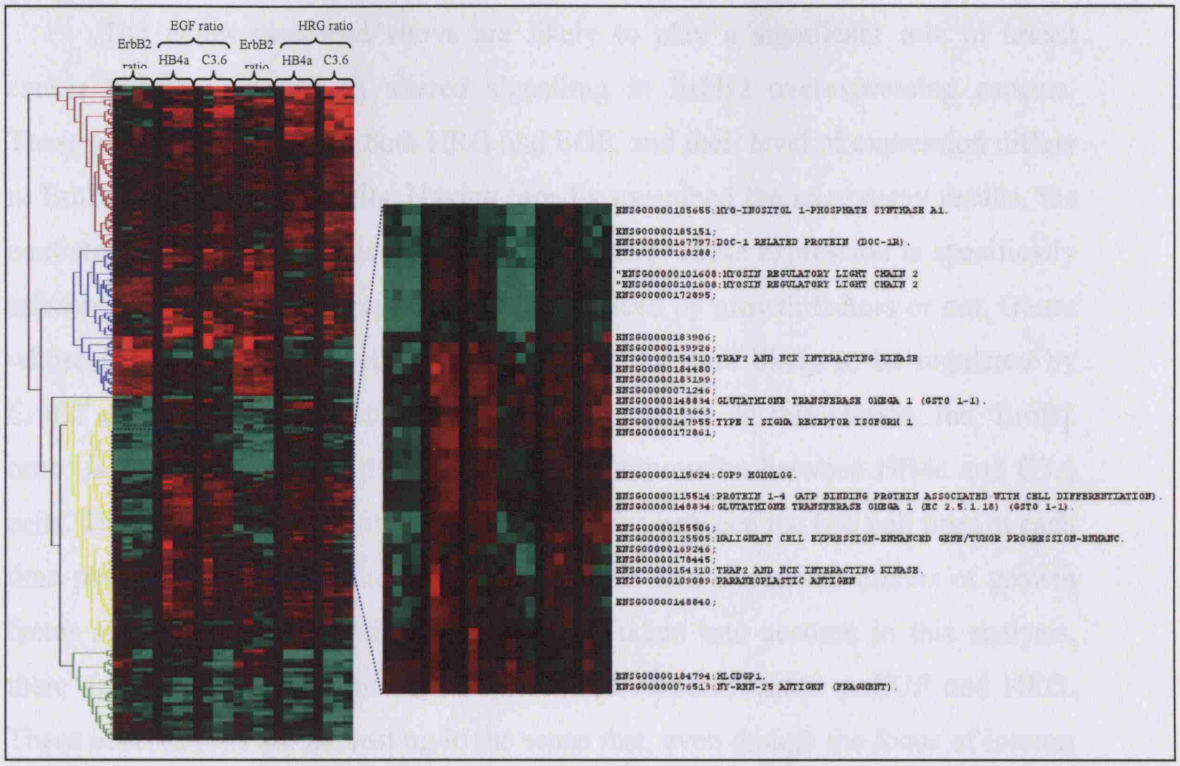

Figure 3.11: Hierarchical clustering of genes with no "common name" annotation. Of the 775 significantly changing genes identified, 197 had no "common name" annotation. Of these, 88 had Ensembl identification numbers (50 of these also had Ensembl gene descriptions), indicating that they could indeed represent annotated sequences. These 197 genes were clustered together to try and find unknown genes whose expression pattern is similar to other known Ensembl genes. Four main clusters were formed, and these are indicated by different colours in the dendogram branches on the left. The cluster on the right shows an enlarged section of the main cluster, indicated by a blue dotted box.

3.5 *ErbB2-Dependent Changes in Gene Expression*

The genes discussed above are likely to play a significant role in breast cancer progression through altered signalling in ErbB2 overexpressing cancers. These genes are induced by both HRG and EGF, and their level of expression differs in ErbB2 overexpressing cells. Having broadly examined the microarray results, the next step in the data analysis was to identify genes whose expression is specifically affected by ErbB2 overexpression, whether induced by growth factors or not. Genes whose expression differs between the two cell lines are likely to be regulated by ErbB2 and may represent important therapeutic targets. In this section, the 'ErbB2 ratio' is used to determine differences in gene expression between HB4a and C3.6 cells. The number of differentially expressed genes between the two cell lines at each time point is shown in Figure 3.12. Although the time-dependent effects of growth factor stimulation on individual genes are not taken into account in this analysis, there are cell line-dependent differences in the responsiveness to EGF and HRG. These observations are interesting in the sense that, even though the focus of interest at this stage is the effect of high ErbB2 levels rather than growth factor treatment, ErbB2 overexpression alters the way cells convey signals and genes are expressed differentially in response to growth factor in ErbB2 overexpressing cells. These differences in gene expression may well give ErbB2-positive cells their proliferative advantage (Timms *et al.* 2002) and promote tumour formation.

A subset of genes was found to be differentially expressed in C3.6 compared to HB4a cells in samples stimulated with EGF and HRG (Figure 3.12). These findings suggest once again that the cell lines show differences in growth factor responsiveness. The difficulty in understanding growth factor response stems from the fact that, using this analysis setup, it is not possible to obtain any information of the directionality of expression changes. For instance, if a gene is up-regulated in HB4a, but to a lower extent in C3.6, one could wrongly conclude that the gene is down-regulated in C3.6 cells because it is not responding to growth factor treatment. Alternatively, if a gene is responsive to growth factor in both cell lines to a similar extent, during statistical analysis this gene would not be considered significantly changing when the two cell lines are compared directly and it may be believed that this gene is not growth factor-responsive. For this reason, the next Section will address changes in gene expression associated with growth factor-responsiveness in each cell line individually, independently of ErbB2, thereby allowing a more

comprehensive picture of growth factor signalling to be obtained. Nevertheless, these results suggest that fundamental differences in signal transduction exist in cells which express higher levels of ErbB2.

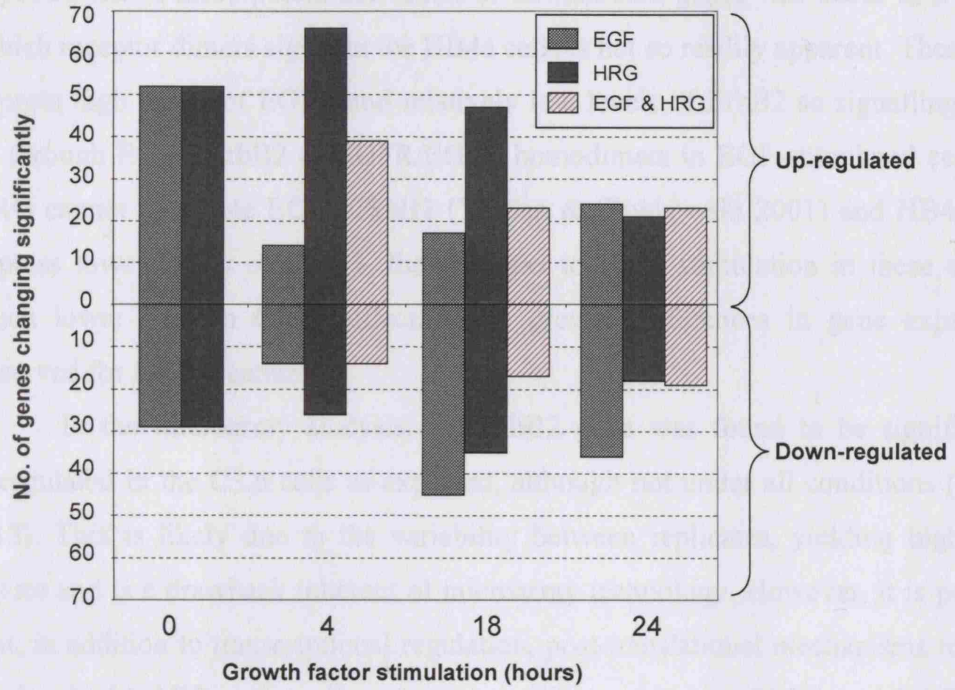

Figure 3.12: Number of genes differentially expressed in C3.6 cells compared to HB4a cells. SAM analysis was carried out to identify differentially expressed genes between HB4a and C3.6 cells. These genes represent the 'ErbB2 ratio' but expression is not exclusively affected by ErbB2, as some of the genes represented in the graph may also be affected by growth factor treatment.

A larger number of genes were found to be differentially expressed following HRG treatment when compared with EGF, particularly at the 4h time point. These findings are similar to the cluster in Figure 3.5 where a number of genes could be seen to be upregulated by HRG in C3.6 cells but not HB4a (red band). As mentioned above, this observation may be explained by the effects of specific receptor dimer formation in response to different ErbB ligands. Given that neither cell line expresses ErbB4 and that ErbB2 is the preferred dimerization partner for the other ErbB receptors, it is expected that cells treated with HRG will signal almost exclusively

through ErbB2/ErbB3 heterodimers in C3.6 cells, which have very high levels of ErbB2 and ErbB3 and lower levels of EGFR compared to HB4a (Figure 3.1 and (Timms *et al.* 2002)), possibly explaining the induction of more genes in the HRG-treated C3.6 cells. In addition, ErbB2/ErbB3 heterodimers are thought to result in the most potent proliferative signal (Pinkas-Kramarski *et al.* 1996) and consequently it is expected that a more potent activation of downstream genes will occur as a result. Which receptor dimers signal in the HB4a cells is not so readily apparent. These cells express high levels of EGFR and relatively low levels of ErbB2 so signalling could be through EGFR/ErbB2 or EGFR/EGFR homodimers in EGF-stimulated cells. As HRG cannot stimulate EGFR/ErbB2 (Yarden & Sliwkowski 2001) and HB4a cells express lower levels of ErbB3, the response to HRG stimulation in these cells is much lower than in C3.6, reflecting the greater differences in gene expression observed for HRG treatment.

In the microarray analysis, the ErbB2 gene was found to be significantly upregulated in the C3.6 cells as expected, although not under all conditions (Figure 3.13). This is likely due to the variability between replicates, yielding high error values and is a drawback inherent of microarray technology. However, it is possible that, in addition to transcriptional regulation, post-translational mechanisms regulate the level of ErbB2 at the cell surface. As mentioned above, EGFR and ErbB3 also display differences in protein expression levels in these cell lines (Figure 3.1-a). Although the receptor level changes were confirmed in microarray experiments for ErbB3, those for EGFR did not pass the statistical test and so its expressin levels were not found to be differentially expressed between the two cell lines. Thus, it is possible that differential EGFR expression is exclusively due to post-transcriptional regulation and does not involve transcriptional regulation of the EGFR gene. Interestingly, ErbB3 was found to be downregulated following HRG treatment in C3.6 cells, suggesting a mechanism of ligand-induced negative regulation of ErbB signalling via regulation of ErbB3 mRNA levels.

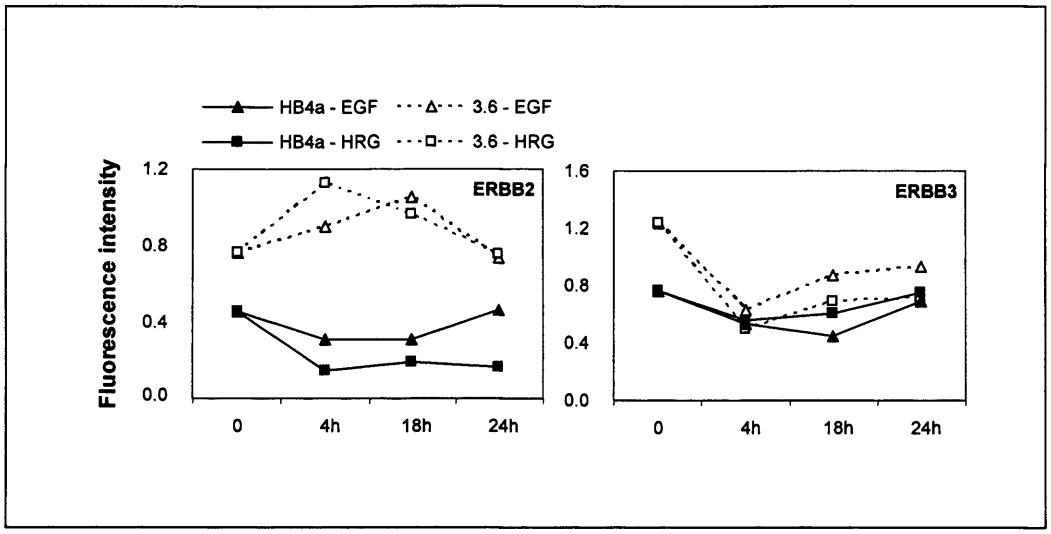

Figure 3.13: Gene expression levels of ErbB2 and ErbB3. Microarray data showed that both ErbB2 and ErbB3 are overexpressed in C3.6 cells. In addition, ErbB3 was significantly downregulated by HRG. EGFR mRNA levels were not significantly changing in any of the conditions examined, suggesting that this receptor is post-transcriptionally regulated.

A total of 309 genes were found to be differentially expressed between HB4a and C3.6 cells at one or more time points of stimulation, with 172 being regulated by ErbB2 overexpression and not by growth factor stimulation (Figure 3.7-a). While many of these genes are known to have roles in cellular transformation, some genes have not been previously implicated in cancer development and/or progression. A complete list of genes that were significantly changing in C3.6 cells relative to HB4a at all time points can be found in Appendix 1. Genes whose expression differed significantly in serum-starved conditions are shown in Table 3.2. Of these, 40 were upregulated and 21 were downregulated. Up-regulated genes include genes involved in cellular metabolism and ATP synthesis (ALDH1A3, COX6C, PKM2, ATP5G3, ATP6V1F, ATP5G1, AKR1B1), possibly reflecting the higher proliferative rate of C3.6 cells compared to HB4a. These genes are discussed in more detail in Section 3.7.6. Genes previously implicated in oncogenesis and regulation of the cell cycle, such as AGR2, AKAP13, EMP1 and BCL6, were also upregulated in C3.6 cells. Among the downregulated genes, a number of interferon (IFN)-inducible genes, such as G1P2, IFITM1 and IFITM2 were apparent. The downregulation of a number of members of the IFN signalling pathway is of interest in this context as IFNs are

known to elicit anti-proliferative responses (Sangfelt *et al.* 2000). This is further discussed in Section 3.7.8 and Chapter 5.

Two members of the annexin family were differentially regulated in serum-starved C3.6 cells, but showed opposite patterns of gene expression. Annexin A1 (ANXA1) was downregulated in C3.6 cells, whereas annexin A2 (ANXA2) was upregulated. Moreover, both annexin genes were found to be upregulated by EGF (Figure 3.14). Annexin A5 was also identified and was significantly downregulated by HRG in both cell lines. The annexins comprise a family of more than 160 calcium-dependent phospholipid-binding proteins and have been implicated in a multitude of cellular processes, including modulation of phospholipase A2 (PLA_2) activity and inflammation (Vishwanath *et al.* 1992), proliferation (Schlaepfer & Haigler 1990), differentiation (Violette *et al.* 1990), vesicular trafficking (Creutz 1992) and intracellular signal transduction (Biener *et al.* 1996). Both annexin A1 and A2 have been identified as major substrates for phosphorylation on tyrosine by EGFR, implicating these proteins in the signalling pathways known to be involved in cancer. Annexin A1 has been reported to be strongly upregulated in a prostate cancer cell line (Vaarala *et al.* 2000), a stomach cancer cell line (Sinha *et al.* 1998), mammary adenocarcinomas (Pencil & Toth 1998) and hepatocarcinomas (de Coupade *et al.* 2000), but was markedly downregulated in prostate cancers and esophageal carcinomas (Paweletz *et al.* 2000,Xia *et al.* 2002b), gastric carcinomas (Hippo *et al.* 2001) and head and neck squamous cell carcinoma (Pedrero J.M.G. *et al.* 2005). Thus, annexin A1 is a promising candidate as a molecular marker of cancer, although the contradictory findings between tumour samples and cell lines require further investigation. Annexin A2 was reported to be overexpressed in drug-resistant small-cell lung cancer cell lines (Cole *et al.* 1992), pancreatic tumour cell lines and primary pancreatic tumours (Vishwanatha *et al.* 1993), and in astrocytic brain tumours (Roseman *et al.* 1994). Intracellular annexin A2 has been implicated in the control of cell proliferation and differentiation. It is tyrosine phosphorylated on activation of the insulin, EGF and PDGF receptors (Karasik *et al.* 1988,Brambilla *et al.* 1991) and represents an in vitro substrate of both protein kinase C (Gould *et al.* 1986,Barnes *et al.* 1991) and $pp60^{v\text{-}src}$ kinase (Radke *et al.* 1980). Thus, annexin A2 also appears to be an important contributor to cancer development.

GENES UP-REGULATED IN SERUM-STARVED C3.6 CELLS				
Symbol	**Ensembl Number and Description**	**GO Biological Process**	**Fold Change**	**t-test**
ALDH1A3	ENSG00000184254:ALDEHYDE DEHYDROGENASE 6	GO:0006629:lipid metabolism	5.45	2.90×10^{-3}
KRT15	ENSG00000171346:KERATIN, TYPE I CYTOSKELETAL 15	GO:0008544:epidermal differentiation	4.66	7.67×10^{-4}
AGR2	ENSG00000106541:ANTERIOR GRADIENT 2	GO:0007048:oncogenesis	4.00	0.01
NCKAP1	ENSG00000061676:NCK-ASSOCIATED PROTEIN 1 (NAP 1)	GO:0006915:apoptosis	3.87	3.90×10^{-3}
COX6C	ENSG00000164919:CYTOCHROME C OXIDASE POLYPEPTIDE VIC	GO:0006091:energy pathways	3.37	0.01
PSCA	ENSG00000167653:PROSTATE STEM CELL ANTIGEN PRECURSOR		2.94	1.82×10^{-4}
KRT13	ENSG00000171401:KERATIN, TYPE I CYTOSKELETAL 13	GO:0008544:epidermal differentiation	2.69	2.25×10^{-3}
YWHAZ	ENSG00000164924:14-3-3 PROTEIN ZETA/DELTA		2.47	0.01
S100P	ENSG00000163993:S-100P PROTEIN		2.45	0.08
PKM2	ENSG00000067225:PYRUVATE KINASE, MUSCLE	GO:0006096:glycolysis	2.41	3.59×10^{-4}
TRAM1	ENSG00000067167:TRAM PROTEIN	GO:0006605:protein targeting	2.38	0.01
ATP5L	ENSG00000167283:ATP SYNTHASE G CHAIN, MITOCHONDRIAL	GO:0015992:proton transport; GO:0006754:ATP biosynthesis	2.26	1.22×10^{-3}
SLC7A7	ENSG00000155465:Y+L AMINO ACID TRANSPORTER 1	GO:0006832:small molecule transport	2.25	0.04
SSBP1	ENSG00000106028:SINGLE-STRANDED DNA-BINDING PROTEIN	GO:0006260:DNA replication	2.25	0.01
SERF2	ENSG00000140264:SMALL EDRK-RICH FACTOR 2		2.21	3.81×10^{-3}
NEDD9	ENSG00000111859:ENHANCER OF FILAMENTATION 1 (HEF1)	GO:0007155:cell adhesion; GO:0000074:regulation of cell cycle	2.18	0.01
SCAMP2	ENSG00000140497:SECRETORY CARRIER-ASSOCIATED MEMBRANE PROTEIN 2	GO:0006886:intracellular protein transport	2.12	0.01
EMP1	ENSG00000134531:EPITHELIAL MEMBRANE PROTEIN-1	GO:0007048:oncogenesis; GO:0008283:cell proliferation	2.10	0.05
LCP1	ENSG00000136167:L-PLASTIN	GO:0000004:biological_process unknown;	2.09	0.01
MGST1	ENSG00000008394:MICROSOMAL GLUTATHIONE S-TRANSFERASE 1		2.06	0.02
DUT	ENSG00000128951:DEOXYURIDINE 5'-TRIPHOSPHATE NUCLEOTIDOHYDROLASE	GO:0006260:DNA replication	2.05	1.20×10^{-3}
DDX5	ENSG00000108654:PROBABLE RNA-DEPENDENT HELICASE P68	GO:0016049:cell growth	2.00	0.01
SRP14	ENSG00000140319:SIGNAL RECOGNITION PARTICLE 14 KDA PROTEIN	GO:0006605:protein targeting	1.98	3.50×10^{-3}
ANXA2	ENSG00000183059:ANNEXIN II (LIPOCORTIN II)	GO:0001501:skeletal development	1.95	0.01
PBP	ENSG00000089220:PHOSPHATIDYLETHANOLAMINE-BINDING PROTEIN		1.80	0.04
ATP5G3	ENSG00000154518:ATP SYNTHASE LIPID-BINDING PROTEIN	GO:0006091:energy pathways; GO:0015992:proton transport	1.78	4.08×10^{-3}
SRI	ENSG00000075142:SORCIN (22 KDA PROTEIN)	GO:0007517:muscle development	1.77	0.03
RPL17	ENSG00000141618:60S RIBOSOMAL PROTEIN L17	GO:0006412:protein biosynthesis	1.71	0.01
AKR1B1	ENSG00000085662:ALDOSE REDUCTASE	GO:0005975:carbohydrate metabolism	1.66	0.06
ATP6V1F	ENSG00000128524:VACUOLAR ATP SYNTHASE SUBUNIT F	GO:0015992:proton transport; GO:0006754:ATP biosynthesis	1.66	0.01
ATP5G1	ENSG00000159199:ATP SYNTHASE LIPID-BINDING PROTEIN	GO:0015992:proton transport	1.64	4.11×10^{-3}
UBN1	ENSG00000118900:UBINUCLEIN 1		1.63	0.02
FTH1	ENSG00000167996:FERRITIN HEAVY CHAIN	GO:0008283:cell proliferation; GO:0006826:iron transport	1.62	0.01
ST14	ENSG00000149418:SUPPRESSOR OF TUMORIGENICITY 14	GO:0006508:proteolysis and peptidolysis	1.62	0.04
PHB	ENSG00000167085:PROHIBITIN	GO:0008151:cell growth and/or maintenance	1.59	0.01
CLTC	ENSG00000141367:CLATHRIN HEAVY CHAIN 1	GO:0006886:intracellular protein transport	1.51	0.01
APLP2	ENSG00000084234:AMYLOID-LIKE PROTEIN 2 PRECURSOR	GO:0007186:G-protein coupled receptor protein signaling pathway	1.48	0.01
HSBP1	ENSG00000166530:HEAT SHOCK FACTOR BINDING PROTEIN 1	GO:0000122:negative regulation of transcription from Pol II promoter	1.48	0.02
FVT1	ENSG00000119537:FOLLICULAR VARIANT TRANSLOCATION PROTEIN 1 PRECURSOR	GO:0008152:metabolism;GO:0007048:oncogenesis	1.45	0.01
BCL6	ENSG00000113916:B-CELL LYMPHOMA 6 PROTEIN	GO:0008284:positive regulation of cell proliferation	1.43	4.18×10^{-3}

GENES DOWN-REGULATED IN SERUM-STARVED C3.6 CELLS				
Symbol	**Ensembl Number and Description**	**GO Biological Process**	**Fold Change**	**t-test**
G1P2	*ENSG00000182106:UBIQUITIN CROSS-REACTIVE PRECURSOR*	GO:0006955:immune response; GO:0007267:cell-cell signaling	0.17	$3.83x10^{-3}$
IFITM1	*ENSG00000185885:INTERFERON-INDUCED TRANSMEMBRANE PROTEIN 1*	GO:0006955:immune response; GO:0008285:negative regulation of cell proliferation	0.25	0.01
IGFBP3	*ENSG00000146674:INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 3 PRECURSOR*	GO:0007165:signal transduction; GO:0001558:regulation of cell growth	0.31	0.01
IFITM2	*ENSG00000185201:INTERFERON-INDUCED TRANSMEMBRANE PROTEIN 2*	GO:0006955:immune response	0.34	0.01
SERPINH1	*ENSG00000149257:COLLAGEN-BINDING PROTEIN 2 PRECURSOR*	GO:0006950:response to stress	0.39	$1.31x10^{-3}$
WFDC2	*ENSG00000101443:MAJOR EPIDIDYMIS-SPECIFIC PROTEIN E4 PRECURSOR*	GO:0006508:proteolysis and peptidolysis	0.43	0.02
TYMS	*ENSG00000176890:THYMIDYLATE SYNTHASE*	GO:0006139:nucleobase, nucleoside, nucleotide and nucleic acid metabolism	0.44	0.01
HDLBP	*ENSG00000115677:VIGILIN (HIGH DENSITY LIPOPROTEIN-BINDING PROTEIN)*	GO:0006869:lipid transport; GO:0008203:cholesterol metabolism	0.50	$1.42x10^{-3}$
RPN1	*ENSG00000163902:DOLICHYL-DIPHOSPHOOLIGOSACCHARIDE--PROTEIN GLYCOSYLTRANSFERASE*	GO:0006464:protein modification	0.51	0.01
GSTP1	*ENSG00000084207:GLUTATHIONE S-TRANSFERASE P*	GO:0007417:central nervous system development	0.51	$7.57x10^{-4}$
RPL37A	*ENSG00000138414:60S RIBOSOMAL PROTEIN L37A*	GO:0006412:protein biosynthesis	0.52	$2.11x10^{-3}$
PLD3	*ENSG00000105223:SIMILAR TO VACCINIA VIRUS HINDIII K4L ORF*	GO:0008152:metabolism	0.53	$2.88x10^{-3}$
FXR1	*ENSG00000114416:FRAGILE X MENTAL RETARDATION SYNDROME RELATED PROTEIN 1*	GO:0006915:apoptosis	0.56	0.01
ANXA1	*ENSG00000135046:ANNEXIN I*	GO:0006928:cell motility; GO:0006629:lipid metabolism	0.57	0.05
SF3B1	*NSG00000115524:SPLICING FACTOR 3B SUBUNIT 1*	GO:0006371:mRNA splicing	0.58	0.02
GMPS	*ENSG00000163655:GMP SYNTHASE*	GO:0006164:purine nucleotide biosynthesis	0.62	$3.60x10^{-3}$
PPP1CC;PPP1CA	*ENSG00000172531:SERINE/THREONINE PROTEIN PHOSPHATASE PP1-ALPHA 1 CATALYTIC SUBUNIT*	GO:0006470:protein amino acid dephosphorylation	0.65	0.04
UBL1	*ENSG00000116030:UBIQUITIN-LIKE PROTEIN SMT3C PRECURSOR*	GO:0006281:DNA repair	0.65	0.01
NPC2	*ENSG00000119655:EPIDIDYMAL SECRETORY PROTEIN E1 PRECURSOR*	GO:0000004:biological_process unknown	0.65	0.01
SHMT2	*ENSG00000182199:SERINE HYDROXYMETHYLTRANSFERASE*	GO:0006520:amino acid metabolism	0.68	0.02
RAB7	*ENSG00000075785:RAS-RELATED PROTEIN RAB-7*	GO:0007264:small GTPase mediated signal transduction; GO:0006897:endocytosis	0.69	0.02

Table 3.2: Genes differentially expressed in C3.6 cells versus HB4a under serum-starved conditions. Genes represent statistically significant changes in gene expression when C3.6 and HB4a cells were directly compared in SAM at T0. Red genes were up-regulated whereas green genes were down-regulated. Fold changes represent the ratio of the average fluorescence intensities of C3.6/HB4a. T-tests were calculated in excel using a two-tailed distribution.

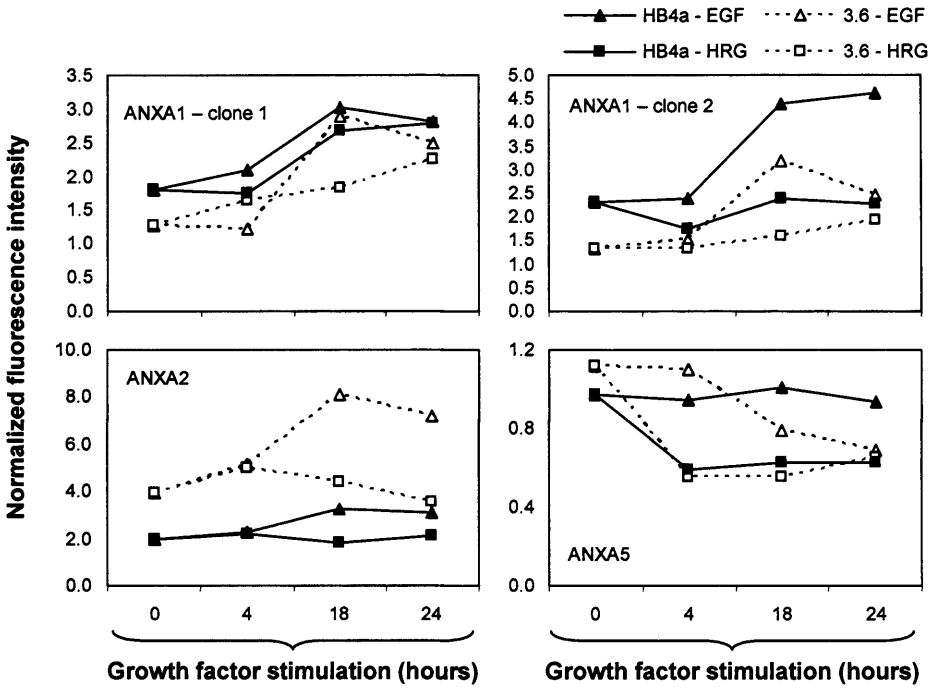

Figure 3.14: Gene expression pattern of the annexin family of proteins. Normalized fluorescence intensity representing expression levels of the annexin family. Annexin A1 was represented twice on the array, with the two represented clones showing similar patterns of gene expression. Both annexin A1 and A2 showed statistically significant differences in expression between HB4a and C3.6 cells at T0.

Features which were not assigned a common name were excluded from Table 3.2. There were 14 up-regulated and 10 down-regulated un-annotated clones, although eight of the up-regulated and six of the down-regulated clones were found to match the sequence of known genes. Up-regulated clones included 248933_A (prohibitin), 249115_A (PDIR, protein disulfide-isomerase A5 precursor) 267484_A (PC4, activated RNA polymerase II transcription cofactor 4) and 306537_A (SDR1, short-chain dehydrogenase/reductase). Three up-regulated clones (183315_A, 795557_A and 795557_B) had sequences that matched human mRNA for an unknown product specific to adipose tissue. Of the downregulated clones, two (113437_A and 296841_A) were found to represent MRCL3 (myosin regulatory light chain 3) and two (212036_A and 249569_A) were found to represent the ribosomal protein RPL24. Thus, sequence alignment for closer inspection of clones in the microarray can reveal genes of known function that may be important in ErbB2-dependent transformation. Nonetheless, there are still sequences which represent unknown genes, and which may indeed prove to be as yet undiscovered genes with important roles in tumourigenesis.

The genes discussed here are likely to be altered due to ErbB2 overexpression and therefore may prove to be important for malignant transformation, possibly mediated by ligand-independent signalling due to ErbB2 overexpression. As discussed above, many genes were also differentially expressed in growth factor-treated C3.6 cells compared to HB4a cells, reflecting the altered signalling properties of the ErbB2-overexpressing cells. Whilst it is not possible to discuss all of these genes individually, a number of them are covered further in Section 3.7, where differentially expressed genes are grouped according to function and their potential roles in ErbB-mediated transformation and/or signalling are discussed.

3.6 Growth Factor-Dependent Changes in Gene Expression

Growth factor signalling through the ErbB family is known to elicit a variety of cellular responses and both EGF and HRG have been shown to be important regulators of cell proliferation, differentiation and motility (Menard *et al.* 2004). Such diverse functions of EGF and HRG are thought to be dependent on their ability to activate complex and overlapping signalling networks that ultimately lead to changes in the expression of multiple and specific gene products which regulate these

processes (Reese & Slamon 1997). Thus, it is of interest to identify the genes induced or repressed downstream of EGF and HRG to gain a better understanding of the function of these ligands *in vivo* and in particular in cancer progression.

A previous microarray analysis of HB4a and C3.6 cells in response to HRG treatment was recently published by our laboratory (White *et al.* 2004). In the present study, these experiments were extended to analyse and compare the effects of HRG and EGF stimulation. In addition, an alternative experimental design was employed to allow comparison across all samples, as explained above (Section 3.3). In order to analyse EGF and HRG-specific changes in gene expression individually as well as genes responsive to both growth factors irrespective of ErbB2 levels, three gene lists were generated according to the Venn diagram presented in Figure 3.7-a: (i) EGF-only responsive genes (199 genes); (ii) HRG-only responsive genes (269 genes); and (iii) genes responsive to both EGF and HRG (135 genes). Notably, HRG was able to induce/repress the expression of significantly more genes compared to EGF and this may be a reflection of the higher signalling potency of ErbB2/ErbB3 heterodimers which are activated following HRG stimulation (Pinkas-Kramarski *et al.* 1996). The total number of genes changing in response to EGF or HRG in each cell line is shown in Figure 3.15. These genes show significantly different expression levels in one or more time point when compared to serum-starved control cells. A larger number of genes were responsive to EGF than HRG in HB4a cells, whereas C3.6 cells showed a higher number of HRG-responsive genes. Once again, this is likely to be due to differences in ErbB receptor levels in these cells. A larger number of genes were downregulated in C3.6 by both EGF and HRG cells compared to HB4a cells. This may represent a mechanism by which ErbB2 overexpression results in the repression of transcription of genes involved in the negative regulation of proliferation, possibly through feedback loops. Finally, genes whose expression is similarly induced or repressed in both cell lines are genes directly affected by growth factors regardless of ErbB receptor levels and are not likely to participate in ErbB2-mediated cellular transformation per se.

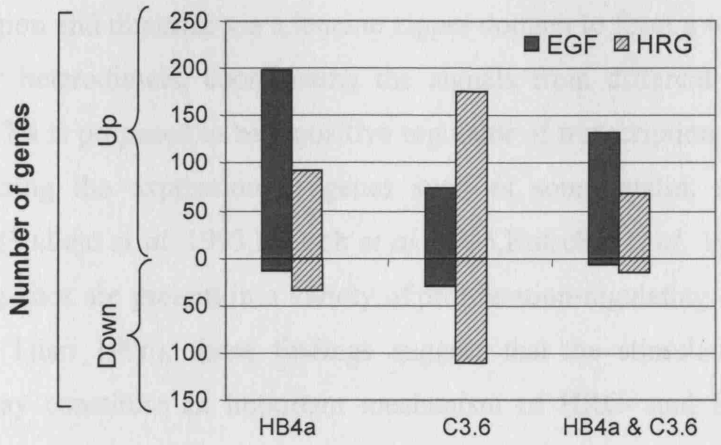

Figure 3.15: Number of genes changing with EGF and HRG stimulation in each cell line. The total number of genes up- or down-regulated by EGF or HRG in HB4a and C3.6 cells alone or in both cell lines is represented. Genes included are those identified by SAM at T4, T18 or T24 compared to T0 control cells. The number of genes changing in this graphical representation does not reflect growth factor specificity and genes changing in EGF-treated samples may also be changing in response to HRG stimulation.

3.6.1 Genes commonly regulated by both EGF and HRG

Genes up- or down-regulated by both EGF and HRG are likely to share common pathways, be activated by the same receptor and/or act downstream of similar adaptor molecules recruited to receptor dimers. To further analyse the expression pattern of genes commonly regulated by both EGF and HRG, *k*-means clustering was used and the genes divided into four groups based on their expression patterns across the time points (Figure 3.16). Genes represented more than once tended to cluster together, thus increasing the confidence in the expression patterns obtained. The first cluster represents a pattern of expression of genes mainly downregulated by growth factor treatment (Figure 3.16-a), and includes PRDX5, GSTP1, TXNIP, ALDH1A3 and MGST3 all of which are genes involved in cellular resistance to oxidative stress. The transcription factor ATF4 was also present in the downregulated cluster, although it did not cluster tightly since its expression was firstly upregulated by EGF and HRG between T0 and T4h (Figure 3.17). This downregulation maybe part of a tight negative feedback loop, where ATF4 is upregulated and than rapidly downregulated again. ATF proteins bind to DNA via

their basic region and dimerize via a leucine zipper domain to form a wide variety of homo- and/or heterodimers, coordinating the signals from different pathways. In particular, ATF4 is proposed to be a positive regulator of transcription (Liang & Hai 1997), increasing the expression of genes such as somatostatin, serotonin and interleukin-2 (Vallejo *et al.* 1993,Bartsch *et al.* 1995,Butscher *et al.* 1998). Because ATF4 binding sites are present in a variety of proliferation-regulating cellular genes (Verrijzer & Tjian 1996), these findings suggest that the stimulation of ATF4 expression may constitute an important mechanism of HRG- and EGF-mediated regulation of genes with diverse functions. Little is known about the regulation of ATF4 expression. ATF4 mRNA is thought to be ubiquitously expressed, whereas the protein is present only at very low levels (Vallejo *et al.* 1993). Upregulation of ATF4 by HRG has been previously reported in MCF-7 cells, and the time-scale was similar to the one observed here (Talukder *et al.* 2000). However, no EGF responsiveness has been reported to date. Further investigation of the regulation of ATF4 by ErbB ligands will provide interesting insights into the role of ATF4 in breast cancer development, and it is possible that ATF4 acts downstream of the ErbB receptor signalling pathway to control proliferation, differentiation and stress response in the mammary epithelium.

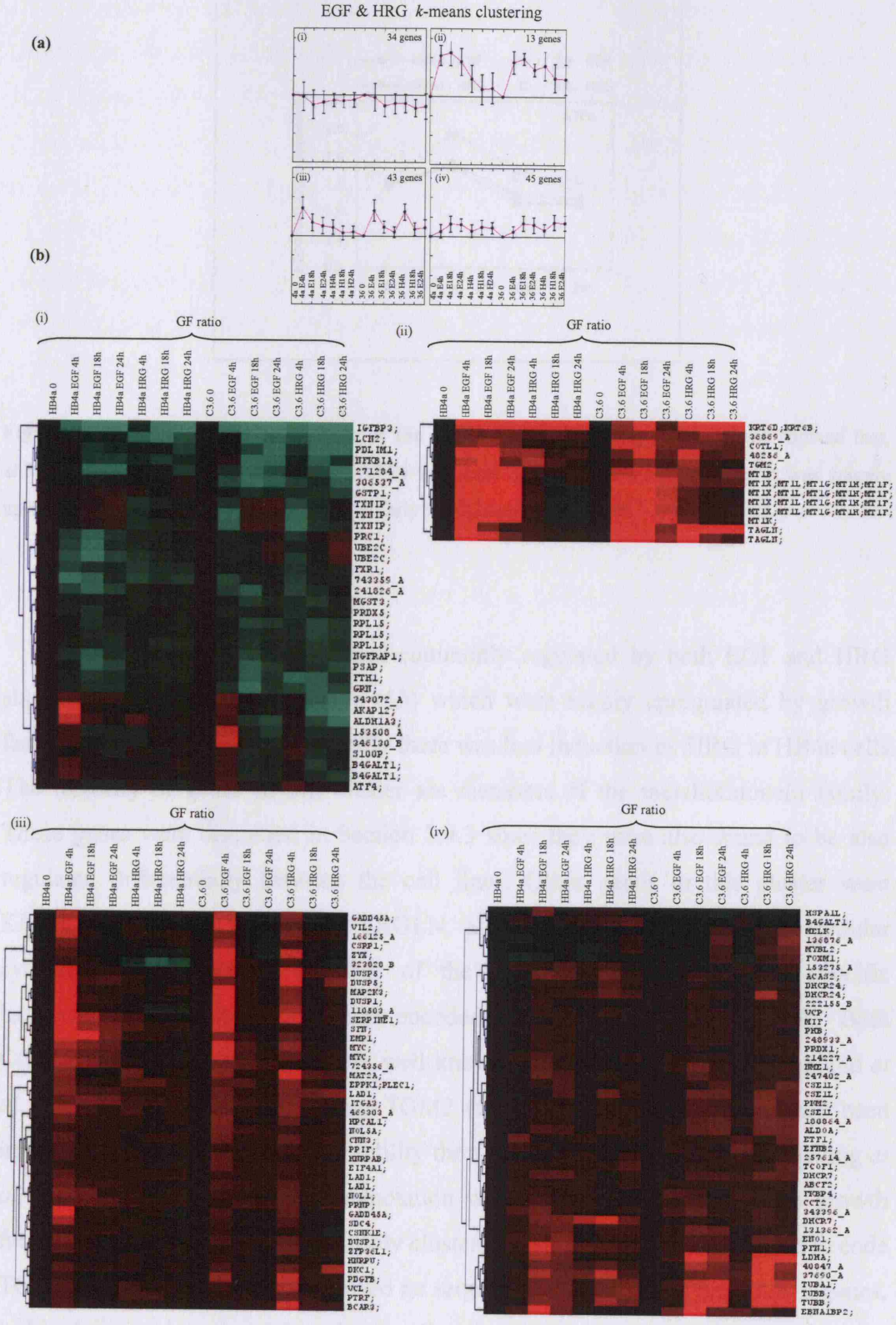

Figure 3.16: K-means clustering of genes differentially regulated by both EGF and HRG. A total of 135 genes identified by SAM to be responsive to both EGF and HRG at one or more time points were divided into four groups according to their gene expression patterns. Each group was then subjected to hierarchical clustering to further group genes with similar expression patterns.

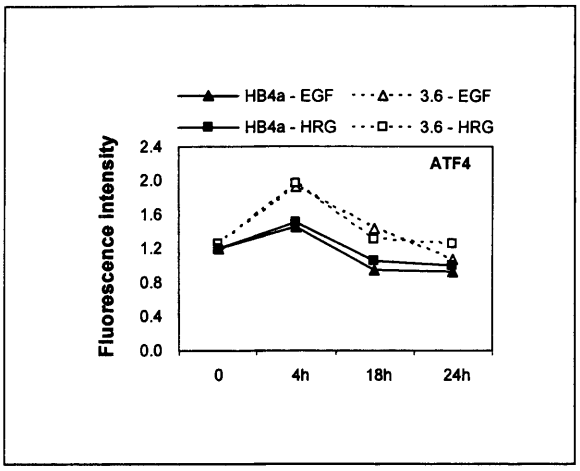

Figure 3.17: Gene expression pattern of the ATF4 gene. Microarray data analysis showed that, although no differences in the level of ATF4 was seen in serum-starved C3.6 cells, it was acutely upregulated by both EGF and HRG, particularly in C3.6 cells.

The second cluster of genes commonly regulated by both EGF and HRG showed a small number of genes (13) which were highly upregulated by growth factor stimulation, although generally there was less induction by HRG in HB4a cells. The majority of genes in this cluster are members of the metallothionein family. These genes were discussed in Section 3.4.3 since they were also found to be also regulated differentially between the cell lines. Other genes in this cluster were KRT6D/B, COTL1, TGM2 and TAGLN, all of which are linked to the cellular cytoskeleton. KRT6 is a member of the keratin family of epithelial-specific intermediate filament (IF) proteins encoded by a large multigene family. Both COTL1 and TGLN (transgelin) are well known actin-binding proteins (Shapland *et al.* 1993,Provost *et al.* 2001), and TGM2 (transglutaminase 2) has recently been implicated as a regulator of cell motility through interaction with integrins (Kang *et al.* 2004). Two clones with no annotation were also highly responsive to growth factors. Clone 48256_A, which closely clustered with TGM2, was also found to code TGM2, while clone 36869_A showed no sequence similarity with any known genes, and its function is completely unknown. Expressed sequences like this are interesting because they represent potentially important components of characterised signalling pathways, and could represent useful novel cancer markers.

Cluster-iii consisted of genes responsive to both EGF and HRG and included genes that were transiently upregulated at T4, but were relatively un-responsive in HRG-treated HB4a cells. This is an interesting finding because it shows enhanced HRG-induced signalling, and consequently proliferation, in ErbB2-overexpressing tumours. Indeed, a number of genes involved in MAPK signalling and downstream targets of this signalling pathway were represented in this cluster, including the protein kinase MAP2K3 (or MEK3), a kinase involved in the activation of the p38 or the stress-activated protein kinase/c-jun N-terminal (SAPK/JNK) stress-response pathways (Roux & Blenis 2004), and DUSP1 and DUSP5, dual-specificity phosphatases which dephosphorylate MAP kinases on both phospho-tyrosine and phospho-threonine residues (Sun *et al.* 1993). Interestingly, SAPK/JNK and p38 MAPK appear to be preferential targets for de-phosphorylation by DUSP1 (Franklin & Kraft 1997). The proto-oncogene MYC and the MYC-inducible protein EMP1, also present in this cluster, are known to be induced in following mitogenic stimulation. In addition, platelet-derived growth factor (PDGFB) was also present in this cluster and it is known to activate signalling pathways that lead to cellular proliferation, survival and migration (Tallquist & Kazlauskas 2004), thus suggesting a mechanism of growth factor-induced growth factor production and signalling potentiation through autocrine mechanisms.

Also present in cluster-iii was SFN (14-3-3 σ). This gene was induced by both growth factors and it has been proposed that it is a p53-dependent negative regulator of G2/M progression (Hermeking *et al.* 1997). The expression pattern of SFN was validated by real time PCR and therefore this gene will be discussed in Chapter 4. GADD45A, also present in this cluster, is another gene thought to induce growth arrest through a p53-dependent mechanism (Zhan 2005), although its induction in these cells where p53 is inactivated would suggest another mechanism of activation and perhaps another function. GADD45 was originally identified as a gene whose mRNA is DNA-damage-inducible and it is thought to play a role in apoptosis via activation of the SAPK/JNK and/or p38 MAPK signaling pathways (Takekawa & Saito 1998). GADD45B, a GADD45-related gene, showed a similar expression pattern, but was only significantly upregulated by EGF and not by HRG. The adhesion and cytoskeletal genes villin 2 (VIL2), zyxin (ZYX), ladinin 1 (LAD1), syndecan 4 (SDC4), integrin-α3 (ITGA3) and vinculin (VCL) were also induced

transiently by EGF and HRG and may be linked to altered adhesion and chemotaxis in response to growth factors.

Finally, cluster-iv represents genes whose expression was upregulated by both EGF and HRG in a similar fashion in both cell lines. Genes involved in cellular proliferation were also present within this cluster, including MYBL2, a MYC-related proto-oncogene encoding a phosphorylated nuclear DNA binding protein that is likely to be involved in transcriptional regulation. MYBL2 expression was higher at 18h and 24h, consistent with its role in the later stages of the cell cycle. Other genes implicated in the regulation of cellular proliferation in this cluster include the forkhead box M1 transcription factor (FOXM1), prohibitin (PHB) and the chromosome segregation gene CSE1L. The clone 248933_A, which clustered tightly with PHB, was also found to code for PHB. A sub-cluster of cluster-iv showed a more potent upregulation in response to growth factor treatment, and contained the TUBB2 and TUBA1, or tubulin β2 and tubulin α1 genes, the structural constituent of microtubules; and two un-annotated clones (40847_A and 37690_A), with high sequence similarity for ATP citrate lyase, a cytosolic enzyme that synthesize acetyl-coenzyme A with a central role in *de novo* lipid synthesis.

This Section broadly outlined genes commonly induced by EGF and HRG, many of which are novel, and showed that a number of these are involved in mitogenic signalling, cell cycle, adhesion and redox and transcription regulation. The next step was to investigate genes whose expression was found to be significantly changing in response to each growth factor individually.

3.6.2 Genes regulated exclusively by EGF

EGF is one of the most widely studied growth factors to date, and has been shown to play a fundamental role in the regulation of cell proliferation, survival, differentiation and development. EGFR knockout in mice results in embryonic through to perinatal death, depending on the genetic background of the host mice (Threadgill *et al.* 1995,Sibilia & Wagner 1995,Miettinen *et al.* 1995). This variable lethality was associated with either placental defects or aberrant epithelial proliferation and/or differentiation in several tissues of neonates, including skin, lung and the gastrointestinal tract. Moreover, surviving neonates suffer from hair and eye defects, respiratory distress and growth retardation. These findings suggest that

EGFR and its ligands have broad roles in development and in a range of ligand-induced cellular activities. By contrast, mice lacking EGF were fertile, showed no histological abnormalities and no differences in growth rate or survival from wild-type mice (Luetteke *et al.* 1999), indicating overlapping or compensatory functions among EGF-like ligands. EGF has been shown to activate a large number of intracellular signalling pathways. It induces the activation of PLCγ to catalyze the formation of two second messengers, namely inositol trisphosphate (IP_3) and diacylglycerol (DG); the activation of Ras proteins, which are key regulators in mitogenesis because they act as intermediates between receptor tyrosine kinases at the plasma membrane and an intracellular cascade of mitogen activated protein kinases (MAPKs) (Bokoch & Der 1993); the activation of the PI3K/Akt pathway (Chen *et al.* 2001); and the phosphorylation of the signal transducers and activators or transcription (STAT) proteins, particularly STAT1, STAT3 and STAT5 (David *et al.* 1996).

In this Section, genes that are modulated by EGF stimulation in normal mammary luminal epithelial cells (HB4a) and in ErbB2 overexpressing cells (C3.6) are discussed. Given the large amount of cellular processes that EGF signalling is involved in, it is not surprising that a large number of genes with diverse functions were identified. A total of 199 genes were found to be significantly up- or down-regulated by EGF over one or more time points. These genes were grouped into four clusters according to their similarity in gene expression determined using *k*-means clustering (Figure 3.18).

Genes downregulated by EGF (cluster-iii) include IGFBP3, whose other clone was present in the cluster of genes which changed with both EGF and HRG. Other downregulated genes include the glycoproteins mucin-1 (MUC1), GPSN2, epithelial membrane protein 2 (EMP2), the cytokinesis-related protein PRC1 and NEDD9, a docking protein that plays a central role coordinating tyrosine kinase-based signalling events related to cell adhesion, and may function in transmitting growth control signals in response to adhesion or growth factor stimulation. (Law *et al.* 1998).

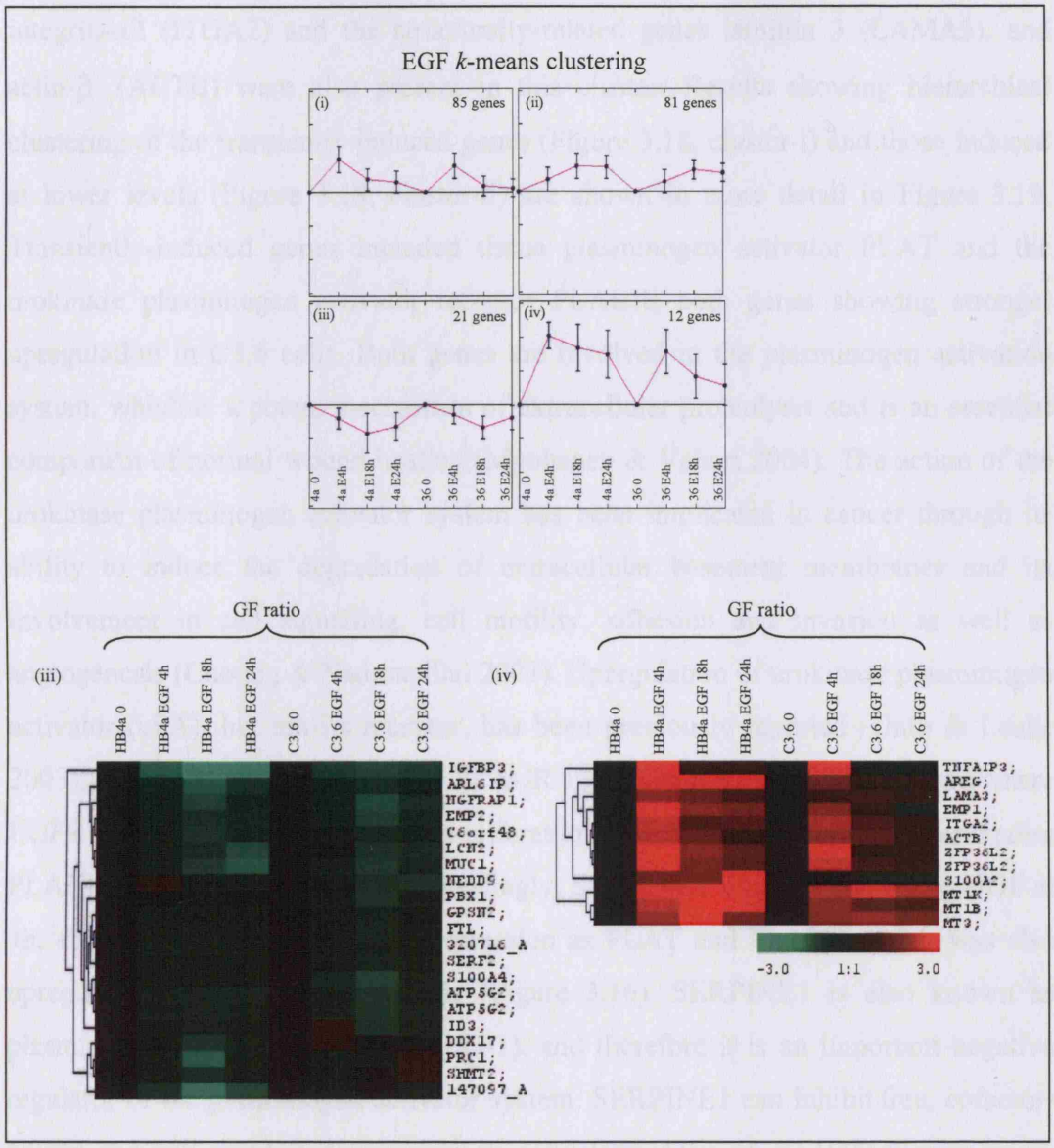

Figure 3.18: K-means clustering of EGF-responsive genes. A total of 199 EGF-responsive genes were identified by SAM. These genes were then divided into four groups using k-means clustering in which the average expression pattern matches its own expression pattern more closely. The average expression pattern and the number of genes assigned to each cluster is shown in the top panel. Each sub-cluster was then subjected to hierarchical clustering, and the results for clusters-iii and -iv are shown in the lower panel.

Among the genes highly upregulated by EGF (cluster-iv) were members of the metallothionein (MT) family MT1K, MT1B and MT3, previously discussed. The EGFR-specific ligand amphiregulin (AREG), the TNFα-induced protein TNFAIP3, integrin-α2 (ITGA2) and the structurally-related genes laminin 3 (LAMA3), and actin-β (ACTB) were also present in this cluster. Results showing hierarchical clustering of the transiently induced genes (Figure 3.18, cluster-i) and those induced at lower levels (Figure 3.18, cluster-ii) are shown in more detail in Figure 3.19. Transiently-induced genes included tissue plasminogen activator PLAT and the urokinase plasminogen activator receptor PLAUR, both genes showing stronger upregulation in C3.6 cells. Both genes are involved in the plasminogen activation system, which is a potent mechanism of extracellular proteolysis and is an essential component of normal wound healing (Myohanen & Vaheri 2004). The action of the urokinase plasminogen activator system has been implicated in cancer through its ability to induce the degradation of extracellular basement membranes and its involvement in cell signalling, cell motility, adhesion and invasion as well as angiogenesis (Choong & Nadesapillai 2003). Upregulation of urokinase plasminogen activator (uPA), but not its receptor, has been previously reported (Unlu & Leake 2003), but links between EGFR and PLAUR signalling have been documented where EGFR can mediate uPA-induced proliferation of malignant cells that overexpress PLAUR (Guerrero *et al.* 2004). Interestingly, SERPINE1 was upregulated by EGF at 4h, showing a similar pattern of expression as PLAT and PLAUR and it was also upregulated by HRG in C3.6 cells (Figure 3.16). SERPINE1 is also known as plasminogen activator inhibtor-1 (PAI-1), and therefore it is an important negative regulator of the plasminogen activator system. SERPINE1 can inhibit free, cofactor-bound and cell-associated plasminogen activators (Ellis *et al.* 1990,Werner *et al.* 1999), and is thought to play a role in ECM remodelling and cellular migration (Potempa *et al.* 1994). Other genes involved in cell adhesion and/or regulation of cellular morphology were present in this sub-cluster, including ITGB4BP (integrin-β4 binding protein), CTNNAL1 (catenin), VASP (vasodilator-stimulated phosphoprotein), CDH3 (cadherin 3) and ARHGEF2 (Rho/Rac guanine nucleotide exchange factor 2). Finally, the TOB2 (transducer of ErbB2-2) gene was present in this cluster. The TOB gene product was recently identified as an ErbB2 binding

protein, although the biological significance of this interaction is still not known (Matsuda *et al.* 1996), and exogenous expression of TOB2 inhibits G1 progression of cells (Ikematsu *et al.* 1999). TOB may be a negative regulator of ErbB2 signalling. TOB2 was upregulated by EGF at 4h only in HB4a cells.

Sub-cluster-ii was composed of genes whose expression was found to be upregulated by EGF at 4h and remained high or went higher at 18 and 24h. Both annexin 1 and annexin 2 (discussed above) were present in this cluster, as well as the S100 family proteins S100A11 and S100A6 (the clone 134924_A was also found be S100A11). S100A2 was also upregulated by EGF and was clustered with the highly upregulated genes in cluster-iv, whereas S100A4 was downregulated by EGF and was thus present in cluster-iii. Finally, S100P was upregulated in serum-starved C3.6 cells, and was significantly upregulated by both EGF and HRG in these ErbB2-overexpressing cells. The calcium-binding S100 protein family is comprised of at least 19 members, and they have been implicated in a variety of cellular processes, including inhibition of protein phosphorylation, regulation of Ca^{2+} homeostasis, regulation of enzyme activity and regulation of the dynamics of cytoskeleton components (Donato 1999). Cytoskeleton-related genes, such as SDC1 (syndecan), TMSB10 (thymosin-β10), ADRM1 (adhesion-regulating molecule 1), PFN2 (profilin 2) and PXN (paxillin, clone 300027_A) were found in this cluster. The relatively large number of genes involved in the regulation of the cytoskeleton and cell adhesion and the widely varied patterns of gene expression observed for such genes suggest that these are highly regulated and complex processes. Successful metastasis formation at distant sites requires tumour cells to complete a number of steps that require changes in the cytoskeleton and in cellular adhesion (Chambers *et al.* 2002). Therefore, the genes identified here may have important roles in ErbB2-induced cancer metastasis. Indeed, the C3.6 cells have been previously shown to display enhanced anchorage-independent growth (Harris *et al.* 1999) and cell adhesion and ECM-related genes were shown to be differentially expressed in these cells in other microarray experiments (Mackay *et al.* 2003,White *et al.* 2004). Consistent with these findings, C3.6 cells were found to be less adhesive than HB4a cells, although adhesion was found to be increased in cells grown on fibronectin-coated plastic or stimulated with HRG (White *et al.* 2004). These findings suggest that ErbB2 can indee alter the adhesive status of cells, and this may have important implications in the progression of breast cancer and metastasis formation.

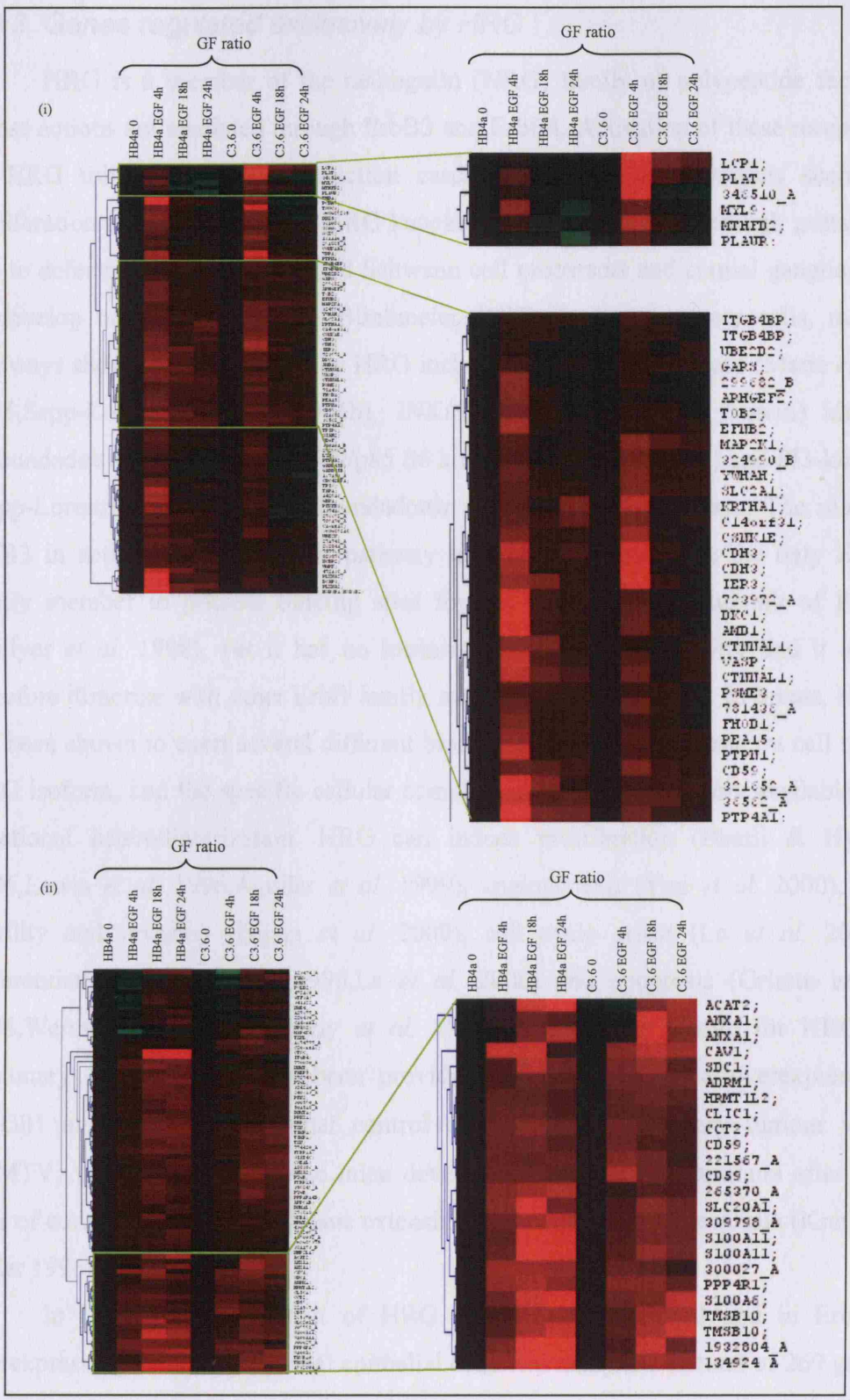

Figure 3.19: Hierarchical clustering of EGF-responsive genes after k-means clustering. Hierarchical clustering of genes responsive to EGF grouped in Figure 3.18. Due to space limitations only part of the clusters can be amplified, but a complete gene list with all EGF-responsive genes can be found in Appendix 1.

3.6.3 Genes regulated exclusively by HRG

HRG is a member of the neuregulin (NRG) family of polypeptide factors whose actions are mediated through ErbB3 and ErbB4. Activation of these receptors by HRG initiates signal transduction cascades involved in processes such as proliferation and differentiation. HRG knockout mice die midway through gestation due to defects in cardiogenesis, and Schwann cell precursors and cranial ganglia fail to develop normally (Meyer & Birchmeier 1995). In breast cancer cells, major pathways shown to be activated by HRG include the ERK/MAP kinase (Marte *et al.* 1995,Sepp-Lorenzino *et al.* 1996b), JNK/SAP (stress activated protein) kinase (Amundadottir & Leder 1998), p70/p85 S6 kinase (Marte *et al.* 1995) and PI3-kinase (Sepp-Lorenzino *et al.* 1996a,Amundadottir & Leder 1998) pathways. The role of ErbB3 in activation of the PI3K pathway is interesting since it is the only ErbB family member to possess binding sites for the p85 regulatory subunit of PI3K (Hellyer *et al.* 1998), yet it has no intrinsic tyrosine kinase activity and it must therefore dimerize with other ErbB family members to be activated. In breast, HRG has been shown to exert several different biological effects that depend on cell type, HRG isoform, and the specific cellular complement of ErbB receptors available for functional heterodimerization. HRG can induce proliferation (Beerli & Hynes 1996,Lewis *et al.* 1996,Aguilar *et al.* 1999), angiogenesis (Yen *et al.* 2000), cell motility and invasion (Hijazi *et al.* 2000), cell cycle arrest (Le *et al.* 2000), differentiation (Jones *et al.* 1996,Le *et al.* 2000) and apoptosis (Grimm *et al.* 1998,Weinstein *et al.* 1998,Daly *et al.* 1999). Support for a role for HRG in mammary carcinogenesis has been provided by transgenic mice overexpressing HRGβ1 under the transcriptional control of the mouse mammary tumour virus (MMTV) promoter, where these mice develop mammary gland tumours after one year of continuous mating and have extensive mammary gland hyperplasia (Krane & Leder 1996).

In this study, the effect of HRG stimulation in normal and in ErbB2-overexpressing mammary luminal epithelial cells was analyzed. A total of 269 genes were found to have significantly changing expression levels following HRG stimulation at one or more time points. These genes were clustered into four groups based on the similarity of their expression levels using *k*-means clustering (Figure

3.20). Of these, 52 were highly downregulated and 39 were highly upregulated by HRG (clusters-i and -iii).

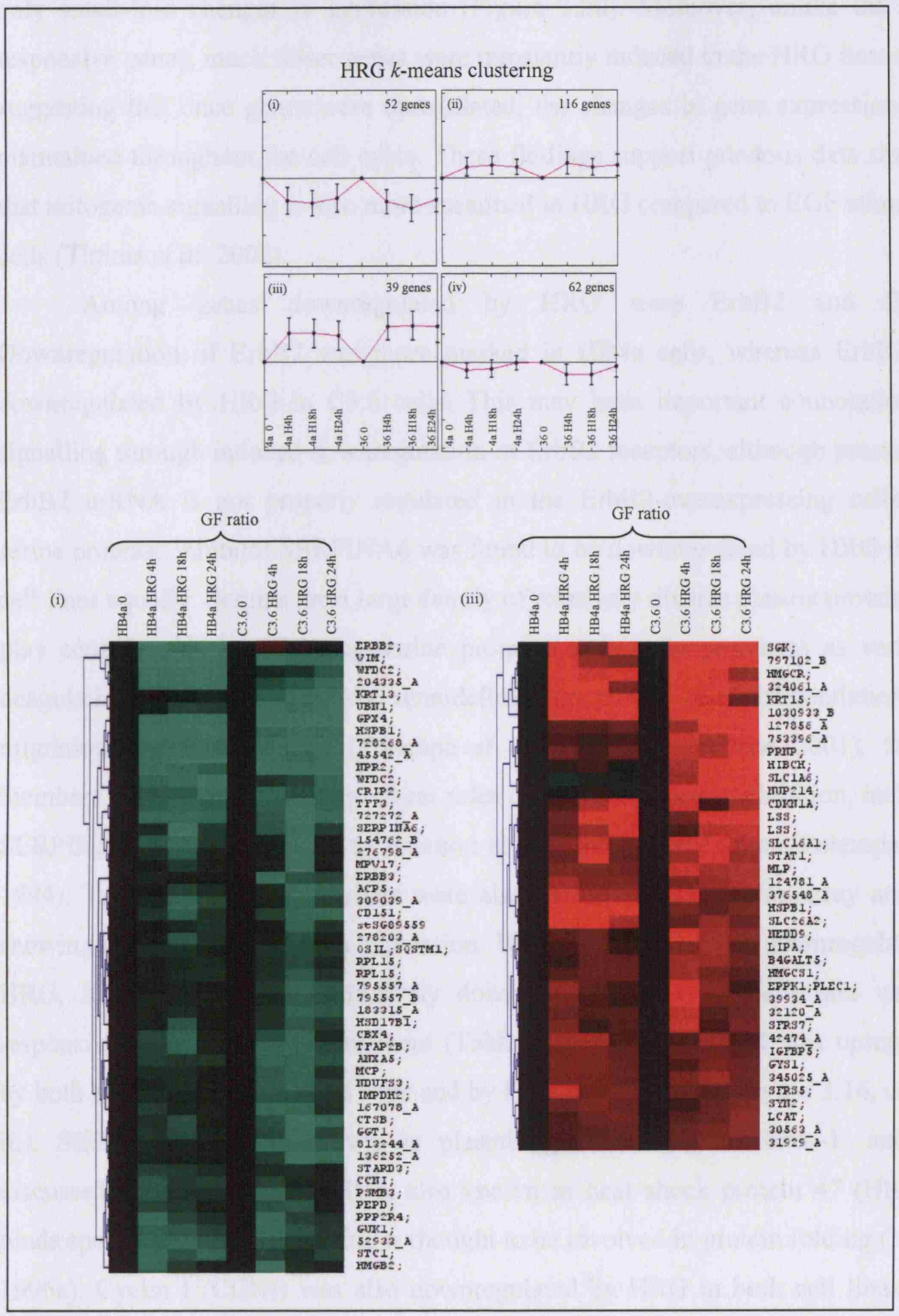

Figure 3.20: K-means clustering of HRG-responsive genes. A total of 260 genes were identified by SAM to be regulated by HRG at one or more time points. These genes were divided into four groups using k-means clustering as in Figure 3.18.

Despite the larger number of HRG-induced genes compared to EGF-induced genes (269 HRG-regulated genes vs. 199 EGF-responsive genes), HRG appeared to result in a weaker induction of gene expression, with the majority of genes showing only small-fold changes in expression (Figure 3.20). Moreover, unlike the EGF-responsive genes, much fewer genes were transiently induced in the HRG time-series, suggesting that once genes were upregulated, the changes in gene expression were maintained throughout the cell cycle. These findings support previous data showing that mitogenic signalling is also more sustained in HRG compared to EGF stimulated cells (Timms *et al.* 2002).

Among genes downregulated by HRG were ErbB2 and ErbB3. Downregulation of ErbB2 was more marked in HB4a cells, whereas ErbB3 was downregulated by HRG in C3.6 cells. This may have important connotations on signalling through induced downregulation of ErbB2 receptors, although presumably ErbB2 mRNA is not properly regulated in the ErbB2-overexpressing cells. The serine protease inhibitor SERPINA6 was found to be downregulated by HRG in both cell lines equally. Serpins are a large family of relatively diverse plasma proteins that play central roles in regulating serine protease activity in processes as varied as coagulation, fibrinolysis, matrix remodelling, apoptosis, cell differentiation, cell migration and inflammation (Potempa *et al.* 1994,Janciauskiene 2001). Several members of the Serpin family perform roles other than protease inhibition, including SERPINA6, which is thought to function as a hormone transporter (Potempa *et al.* 1994). Two other family members were also identified in the microarray analysis, showing marked differences in expression. While SERPINA6 was downregulated by HRG, SERPINH1 was constitutively downregulated in C3.6 cells and was not responsive to growth factor treatment (Table 3.2), and SERPINE1 was upregulated by both EGF and HRG in C3.6 cells and by EGF in HB4a cells (Figure 3.16, cluster-iii). SERPINE1 is also known as plasminogen activator inhibitor-1, and was discussed previously. SERPINH1, also known as heat shock protein 47 (HSP-47), binds specifically to collagen and is thought to be involved in protein folding (Nagata 1996a). Cyclin I (CCNI) was also downregulated by HRG in both cell lines. This gene is a more recent addition to the cyclin family, has functional similarities to cyclins G1 and G2, and may play a role in DNA damage checkpoint control (Bates *et al.* 1996). A few structurally-related genes were downregulated by HRG, including

VIM (vimentin), FAT2 (cadherin family member 8) and DCTN3 (dynactin 3) (the latter two being present in sub-cluster-iv, Figure 3.22).

A number of genes upregulated by HRG in cluster-iii are genes involved in cellular metabolism, such as PRNP, HIBCH, LSS, B4GALT5, HMGCS1, GYS1 and LCAT, possibly reflecting an increase in metabolic rate which occurs when cells are proliferating. IGFBP5 was also present in this cluster and was upregulated by HRG with its expression remaining high at 24h in both cell lines. IGFBP3, another family member was however found to be downregulated in C3.6 cells and by growth factor stimulation. IGFBPs are important regulator of proliferation as they can modulate the interaction of IGFs with their receptors (Ricort 2004). Other genes of interest in this cluster were STAT1 and CDKN1A (p21), both of which are discussed below.

Another interesting gene upregulated by HRG was FOXM1 (present in cluster-iv, data not shown). Two clones of this gene were represented on the array, and they showed very similar expression patterns, although one of them was also changing in response to EGF (Figure 3.21). FOXM1 is a mammalian Forkhead transcription factor which plays a major role in the G2/M transcriptional programme by regulating the expression of a number of G2/M-specific genes such as cyclin B1, cyclin B2, Cdc25B and cyclin A2 (Laoukili *et al.* 2005). It is possible that its upregulation only in the C3.6 cells may reflect the faster cell cycling rate observed in these cell lines. FOXM1 clustered tightly together with two clones that had no annotation (34497_A and 51927_A, Figure 3.22), which also showed an upregulation only in C3.6 cells. Both of these were found to have very high sequence similarity with the gene NIP3, a member of the BCL2/adenovirus E1B 19Kd-interacting protein (BNIP) family. NIP3 contains a BH3 (BCL-2 homology 3) domain which has been associated with a pro-apoptotic function (Chen *et al.* 1997,Yasuda *et al.* 1998). Moreover, NIP3 has been shown to be induced by hypoxia and lead to hypoxia-induced apoptosis in a mechanism dependent on HIF-1 (Bruick 2000). In addition to FOXM1, other cell cycle dependent genes were present in this cluster and were predominantly kinases. TK1 (thymidine kinase) was downregulated by HRG at 4h in both cell lines, as was PRKCL1 (protein kinase N1), but their expression returned to basal levels by the later time points. STK15/6, a mitotic serine/threonine kinase that regulates chromosome segregation in mammalian cells (Nigg 2002),was upregulated by HRG in both cell lines at 24h.

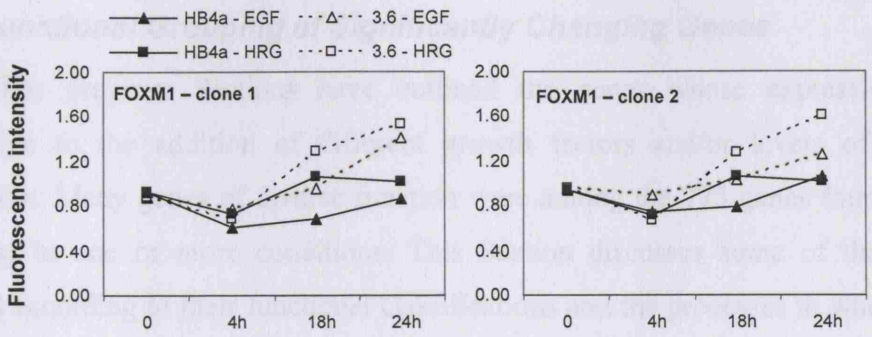

Figure 3.21: Gene expression data for the FOXM1 gene. Microarray fluorescence intensity data is shown for both clones on the array representing the FOXM1 gene.

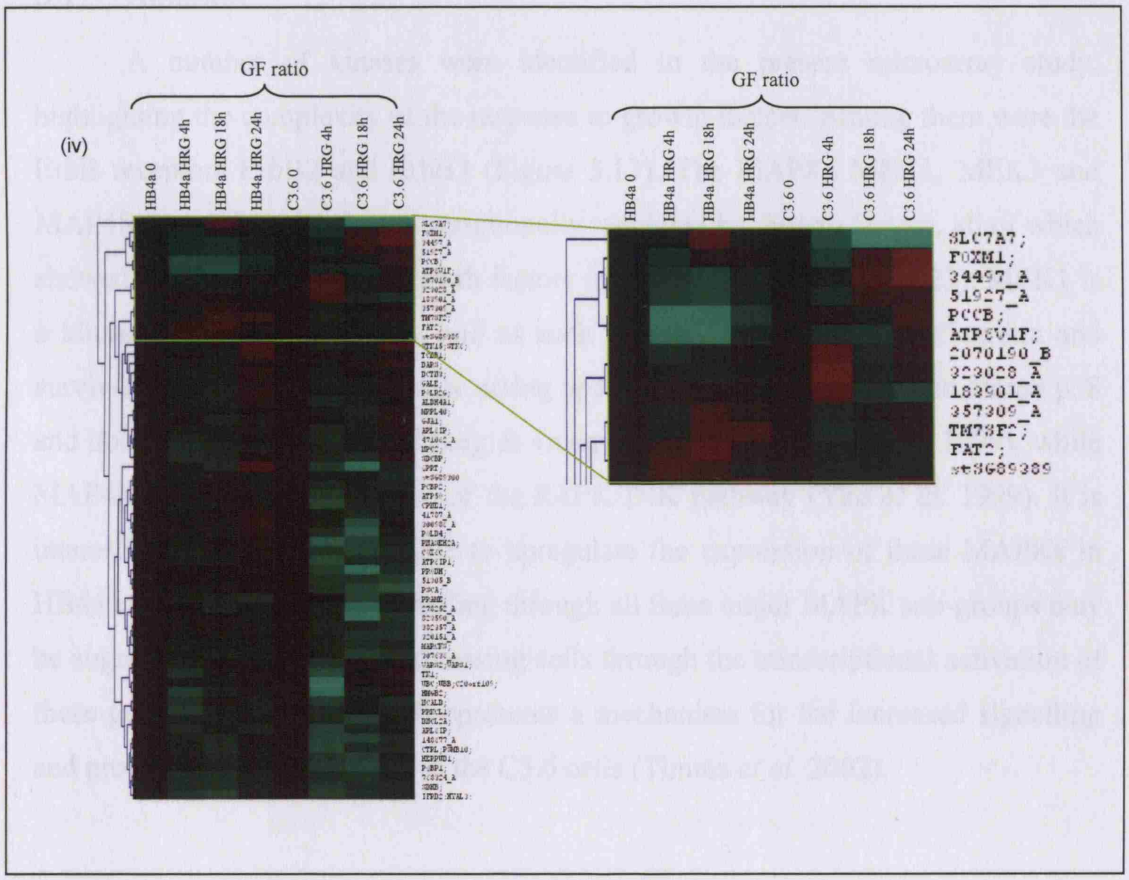

Figure 3.22: Hierarchical clustering of HRG-responsive genes after k-means clustering. Hierarchical clustering of sub-cluster-iv (Figure 3.20).

3.7 Functional Grouping of Significantly Changing Genes

The previous Sections have outlined the genes whose expression was responsive to the addition of different growth factors and/or levels of ErbB2 expression. Many genes of diverse function were among the 775 genes found to be changing in one or more conditions. This Section discusses some of the genes grouped according to their functional classifications and the processes in which they are involved, with special focus on the processes known to be de-regulated in cancer. Although genes of similar function may not be co-regulated under the same conditions, they were all identified as potential mediators or targets of ErbB receptor signalling that might be involved in ErbB2-dependent tumourigenesis.

3.7.1 Kinases

A number of kinases were identified in the present microarray study, highlighting the complexity of the response to growth factors. Among them were the ErbB receptors ErbB2 and ErbB3 (Figure 3.13). The MAPKs MEK1, MEK3 and MAP4K4 were found to be transcriptionally regulated by growth factors, all of which showed higher induction by growth factors in the C3.6 cells (Figure 3.23). MEK1 is a kinase upstream of ERK1/2, and as such is a key mediator of proliferation and survival signals. MEK3 is a kinase acting upstream of the stress activated kinase p38 and does not activate ERKs (Zheng & Guan 1993,Schaeffer & Weber 1999), while MAP4K4 is a specific activator of the SAPK/JNK pathway (Yao *et al.* 1999). It is interesting that HRG was unable to upregulate the expression of these MAPKs in HB4a cells, indicating that signalling through all three major MAPK sub-groups may be augmented in ErbB2 overexpressing cells through the transcriptional activation of these genes, and this probably represents a mechanism for the increased signalling and proliferative rate observed in the C3.6 cells (Timms *et al.* 2002).

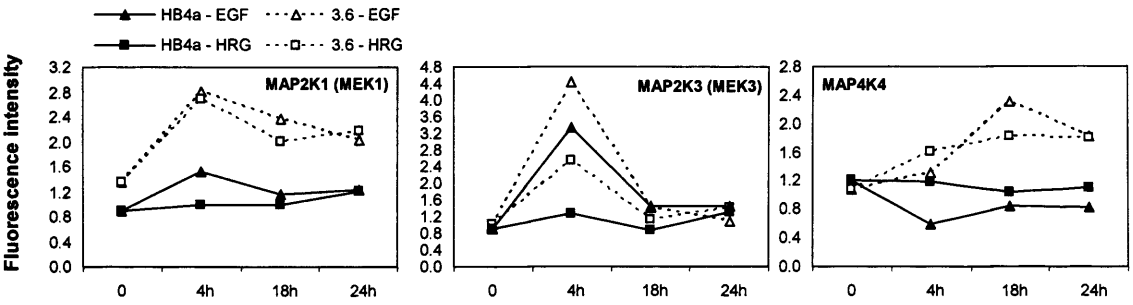

Figure 3.23: Expression profile of MAPK genes. The MAPK genes MEK1, MEK3 and MAP4K4 were identified as significantly changing in response to one or more of the conditions studied, each showing distinct patterns of expression.

The casein kinases 1 (CK1) isoforms α1 and ε (CSNK1A1 and CSNK1E) were both found to be transiently upregulated by EGF in C3.6 and HB4a cells, with one CSNK1E clone also significantly upregulated by HRG in the C3.6 cells. The CK1 family is comprised of at least seven mammalian isoforms (α, β, γ1, γ2, γ3, σ and ε), and their activity is regulated by extracellular stimuli, subcellular localization, interactions with various cellular structures and proteins, as well as autophosphorylation and proteolytic cleavage of their C-terminal regulatory domains. CK1 substrates include a number of cytoskeletal proteins (e.g. myosin, vinculin and dynein), receptors (e.g. PDGF receptor, insulin receptor β, and TNFα receptor) and transcription-related proteins (e.g. p53, RNA polymerase I and II and β-catenin), thereby linking the CK proteins to a wide range of cellular processes (Knippschild *et al.* 2005). Both CK1α and ε have been described as regulators of Wnt- (wingless)-signalling, a pathway which plays an important regulatory role in cell proliferation, through the phosphorylation of the Wnt pathway effector β-catenin (Amit *et al.* 2002,Liu *et al.* 2002a). In addition, CK1 isoforms have been implicated in the regulation of cell division (Gross *et al.* 1997,Behrend *et al.* 2000) and inhibition of apoptosis (Beyaert *et al.* 1995,Izeradjene *et al.* 2004). Thus, CK1 proteins could influence the progression of tumours through many different cellular processes. Although EGF has been reported to induce the phosphorylation of CK proteins (Ackerman *et al.* 1990), there are no reports to date of the ability of this growth

factor to regulate CK transcription and the results presented here provide clues to an additional regulatory mechanism of CK expression.

The ribosomal protein S6 kinase 2 (RPS6KB2, S6K2) was among the genes found to be upregulated in response to ErbB2 overexpression, but was not shown to be responsive to growth factors. S6 kinases are responsible for the phosphorylation of the S6 ribosomal protein, which leads to an increase in the synthesis of components of the protein translational machinery, particularly ribosomal proteins (Duronio *et al.* 1998). It is becoming increasingly recognized that some of the transforming-, proliferation- and survival-promoting effects of PI3K are mediated through the S6K pathway (Blume-Jensen & Hunter 2001). Selective inhibition of mitogen-induced S6K1 activation with neutralizing antibodies or with the immunosuppressant rapamycin inhibits cell growth (Price *et al.* 1992,Chung *et al.* 1992,Lane *et al.* 1993). Indeed, S61K is one of the best carachterized downstream targets and effectors of mTOR (mammalian target of rapamycin), which is itself activated by Akt and is a prime target for therapeutic development against cancer (Rowinsky 2004). S6K2 was cloned based on its sequence homology to S6K1, although its cellular functions and *in vivo* substrate(s) have not yet been established. Initial studies on S6K2 showed that it can phosphorylate S6 *in vitro*, and that upstream kinases known to regulate the catalytic activity of S6K1, such as PI3K, PDK1 and mTOR, may also play a role in S6K2 regulation (Gout *et al.* 1998,Lee-Fruman *et al.* 1999,Koh *et al.* 1999). Thus, it is possible that S6K1 and S6K2 may have common functions in cells. Both S6K1 and S6K2 were found to be upregulated in human breast tumours (Filonenko *et al.* 2004), and S6K2 expression was positively correlated with the expression of the proliferation markers PCNA and Ki-67 in breast adenocarcinomas (Lyzogubov *et al.* 2005), suggesting that S6K2 is involved in the regulation of malignant transformation. The results presented here further reinforce this hypothesis, and provide evidence that ErbB2 is involved in the upregulation of S6K2 in breast cancers.

Other kinases identified in the present study include the Src family kinases FYN (downregulated in C3.6 cells) and LYN (transiently upregulated by EGF in both cell lines), the focal adhesion kinase-1 PTK2 (upregulated by HRG), the cell cycle checkpoint kinase CHEK1 (upregulated in C3.6 cells) and the serum- and glucocorticoid-inducible kinase SGK (transiently upregulated by HRG).

3.7.2 Phosphatases

Protein phosphatase-1 (PP1) is a major eukaryotic Ser/Thr phosphatase that regulates a broad range of cellular processes, including cell cycle progression, transcription, muscle contractility, glycogen metabolism, protein synthesis, and apoptosis (Xu *et al.* 2003,Ceulemans & Bollen 2004). The PP1 holoenzyme is comprised of one of four catalytic subunits which can form complexes with over 50 regulatory subunits, giving PP1 distinct substrate specificities and cellular localizations and allowing the de-phosphorylation of a large number of proteins (Cohen 2002). Here, the mRNA for the PP1 catalytic subunits PPP1CC or PPP1CA or both was found to be downregulated in the ErbB2-overexpressing cells (a single clone on the array represents both genes due to high sequence homology). This finding is interesting as it supports the hypothesis that ErbB2 overexpression leads to increased proliferation and survival signalling through reduced de-phosphorylation of PP1 substrates. Interestingly, it has been recently shown that ErbB2 inhibition promotes PP1-dependent Akt inactivation (Xu *et al.* 2003). Although further studies are needed, the findings presented here suggest a role for ErbB2 in the regulation of PP1.

Other differentially expressed phosphatases and their regulatory subunits included the regulatory subunit of PP2 and PP4 (PPP2R4 and PPP4R1), which were up- and downregulated following HRG treatment, respectively; PPP1R14B, which was responsive to EGF; and the tyrosine specific phosphatases PTP4A1, PTP4A2 and PTPN1, which were upregulated by EGF in both cell lines. The dual specificity phosphatases DUSP1 and DUSP5 were among the genes found to be differentially regulated in response to ErbB2 as well as to both EGF and HRG (Figure 3.7-b). Both DUSP genes were transiently upregulated by EGF in both cell lines and by HRG only in C3.6 cells, and EGF was a more potent inducer of DUSP5 in HB4a cells (Figure 3.24). DUSPs can dephosphorylate and inactivate MAPKs (Sun *et al.* 1993), and their expression is highly induced in response to mitogenic and stress stimuli, thereby providing an auto-regulatory mechanism for signal termination (Keyse & Emslie 1992,Charles *et al.* 1993). DUSP1 has been shown to dephosphorylate all three major MAPK sub-groups (ERK, SAPK/JNK and p38) (Franklin & Kraft 1997), while DUSP5 binds to and inactivates ERK1 and ERK2, but not SAPK/JNK or p38 MAPKs (Mandl *et al.* 2005). A role of DUSP proteins in cancer is beginning to be

established: conditional expression of DUSP1 prevented human lung cancer cells from entering the cell cycle in response to mitogens (Li *et al.* 2003), upregulation of DUSP1, and the consequential dephosphorylation of ERK, was shown to be a pre-requisite for nitric oxide-induced apoptosis in breast cancer cell lines (Pervin *et al.* 2003) and transfection of DUSP5 significantly reduced colony formation of lung cancer cell lines (Ueda *et al.* 2003). Conversely, DUSP1 has been shown to be overexpressed in breast tumours (Wang *et al.* 2003) and in a large-scale RNA interference screen to identify genes that induce apoptosis of HeLa cervical carcinoma cells, a number of DUSPs, including DUSP5, were identified (MacKeigan *et al.* 2005), suggesting that these phosphatases may mediate cell survival. Nonetheless, the results presented here reinforce the fact that DUSPs are key regulators of proliferation and survival signalling, and their differential induction by growth factors in the ErbB2-overexpressing cells suggests that these phosphatases may have an important role in breast cancer signalling.

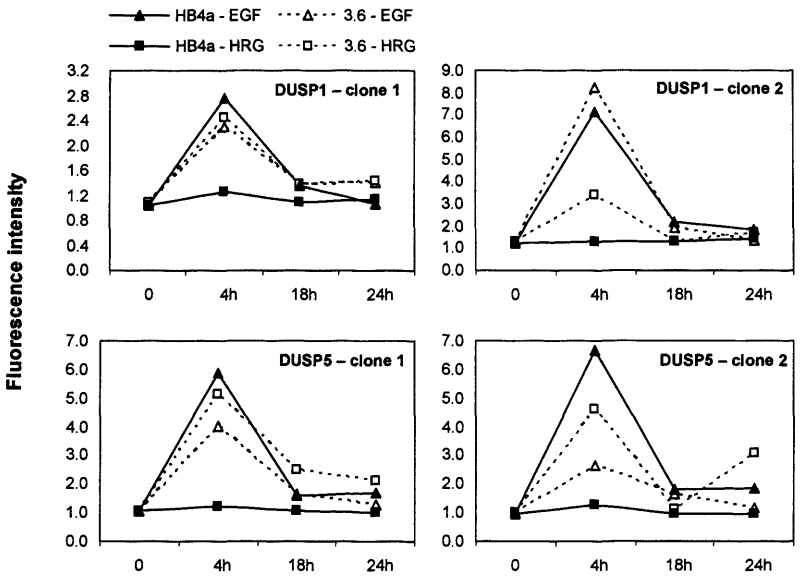

Figure 3.24: Gene expression profile of the DUSP genes. Both DUSP1 (top panel) and DUSP5 (lower panel) were represented twice on the arrays. Their expression profile obtained by microarray is shown here.

3.7.3 Genes involved in the regulation of cell proliferation & survival

Some growth factors and growth factor-related genes were identified in this analysis. IGFBP3, the major carrier protein for insulin-like growth factor (IGF)-1 and IGF-2 in the circulation, has already been discussed above and its expression is further examined by real time PCR in the next Chapter. AREG (amphiregulin), a growth factor originally isolated from conditioned media of the human breast carcinoma cell line MCF-7, belongs to the EGF-like family of growth factors (Shoyab *et al.* 1988,Plowman *et al.* 1990). AREG was significantly upregulated by EGF in HB4a cells, but not C3.6 cells (Figure 3.25). AREG expression was also validated by real-time PCR and will be further discussed in the next chapter. PDGFB (platelet-derived growth factor-β) was among the 59 genes found to be significantly changing in response to ErbB2, EGF and HRG. Its expression was increased at 4h and returned to basal levels by 24h, with a much higher induction observed in the ErbB2-overexpressing cells (Figure 3.25). PDGFB can stimulate various cellular processes including growth, proliferation and differentiation and is one of the principal mitogens found in human serum. In addition to platelets, a number of cell types have been reported to produce PDGF, including endothelial cells, vascular smooth muscle cells and activated macrophages/monocytes (Hughes *et al.* 1996). It would appear that luminal epithelial cells can also produce PDGF. Induction of PDGF secretion by EGF has been reported previously, with PDGF expression being regulated at the transcriptional level (Silver *et al.* 1989). VEGF (vascular endothelial growth factor) was also identified in this analysis, and is of special interest because of its involvement in angiogenesis and tumour metastasis. VEGF was present in the "EGF only" gene list, and was transiently upregulated following EGF treatment, in agreement with previous work (Ferrara *et al.* 2003). Although VEGF has been shown to be induced by HRG in breast cancer cells, resulting in increased endothelial cell migration and angiogenesis (Yen *et al.* 2000,Xiong *et al.* 2001), HRG did not induce its expression significantly. Similarly, despite reports that ErbB2 overexpression can upregulate VEGF expression (Yen *et al.* 2000,Konecny *et al.* 2004,Loureiro *et al.* 2005), the microarray study discussed here showed no difference between HB4a and C3.6 cells. The differential expression of growth factors presented here reflects the important role of autocrine/paracrine feedback loops in the augmentation of ErbB-

mediated signalling, and suggests that this may be a mechanism of ErbB2-mediated transformation.

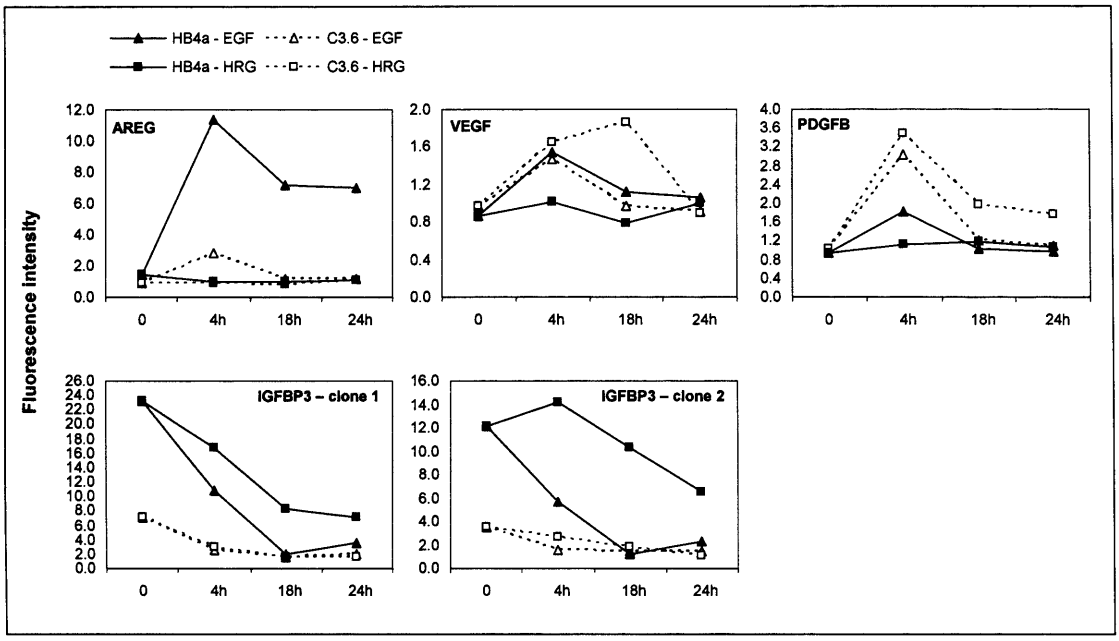

Figure 3.25: Gene expression profile of growth factor-related genes. Gene expression profiles of the growth factors AREG, VEGF and PDGFB and of the growth factor-binding protein IGFBP3 are shown.

Several genes involved in regulating the cell cycle were also found to be significantly changing in response to ErbB2 overexpressiong, EGF and/or HRG treatment. Progression through the cell cycle phases is a highly regulated process, requiring the cooperative action of several classes of cyclin-dependent kinases (CDKs) (Figure 3.26-a). When quiescent cells enter the cycle, D-type cyclins are induced in response to mitogenic signals and assemble with their catalytic partners, CDK4 and CDK6, as cells progress through G1 phase. In G1, an important target of the CDKs is the retinoblastoma protein (pRb). Hypophosphorylated pRb binds to E2F transcription factor, making it unavailable for activation of transcription. G1 cyclin-CDK complexes phosphorylate pRb on multiple sites, thereby releasing E2F and enabling it to participate in the transcription of genes necessary for DNA

synthesis and progression of the cell cycle into S-phase. Cyclin E-CDK2 also becomes activated later in G1 and completes this process by phosphorylating pRb on additional sites. Cyclin A- and cyclin B-dependent kinases (CDK2 and CDK1) are activated later in the cell division cycle and maintain pRb in a hyperphosphorylated form until cells exit mitosis (Schafer 1998,Sherr & Roberts 1999). Negative regulation of the cell cycle is also critical, and is associated with different events, such as binding of CDK inhibitors, cyclin dissociation and phosphorylation or dephosphorylation of CDKs (Figure 3.26-b) (Lee & Yang 2001). Inhibition of the cell cycle in the G2 phase occurs largely by the inhibitory phosphorylation of CDK1 by Wee1/Myt1 (Tourret & McKeon 1996). In the G1 and S phases of the cell cycle, two families of inhibitors are thought to inhibit CDKs. The first class includes the INK4 proteins, which specifically inhibit CDK4 and CDK6, and include p16, p15, p18 and p19 (Roussel 1999). A more broadly acting class of CKIs is the Cip/Kip family, whose actions affect the activities of cyclin D-, A- and E-dependent kinases, and includes p21, p27 and p57 (Sherr & Roberts 1999).

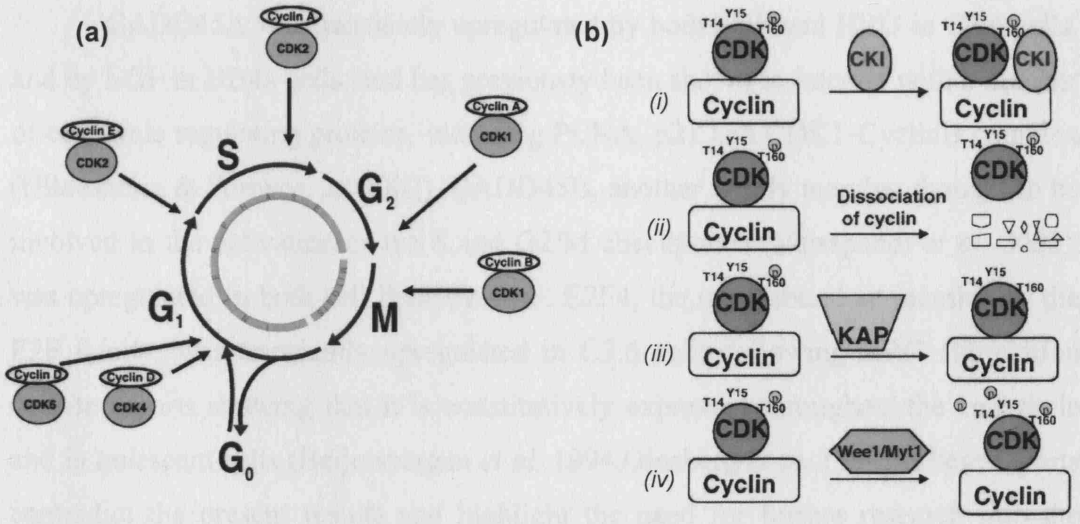

Figure 3.26: Cyclins, CDKs and regulation of the cell cycle. (a) Progression through each phase of the cell cycle is regulated by various CDK-cyclin complexes (Vermeulen et al. 2003). **(b)** Mechanisms that negatively regulate CDKs: (i) binding to CDK inhibitors (CKIs); (ii) dissociation and degradation of cyclins; (iii) dephosphorylation by CDK-associated protein phosphatases (KAP); and (iv) negative regulation of CDKs by phosphorylation of threonine 14/tyrosine 15 residues mediated by Wee1-like kinases (Lee & Yang 2001).

In this study, p21 (CDKN1A) was significantly upregulated following HRG stimulation at 4h in both HB4a and C3.6 cells in agreement with previous work (Timms *et al.* 2002), and it may actually be required for CDK-cyclin assembly (Sherr & Roberts 1999). CHEK1, a protein kinase required for proper arrest of the cell cycle in response to DNA damage (Chen & Sanchez 2004), was upregulated in C3.6 cells compared to HB4a, but only at the 24h time point. Although changes in CHEK1 expression have been reported in cancers, the role of ErbB2 in the regulation of CHEK1 expression has not yet been studied and requires further investigation. CKS2 (Cdc28 protein kinase 2), a protein whose function has yet to be properly established, but is thought to play a role in cell cycle regulation due to it ability to bind cyclin-bound CDK1 (Egan & Solomon 1998), was also upregulated in response to 24h HRG stimulation and was more highly induced in the C3.6 cells. No direct links between ErbB2 and CKS2 have been reported to date, although previous microarray studies have found that ErbB2 and CKS2 were co-expressed in gastric tumours (Hippo *et al.* 2002) and in a "poor prognosis" group of breast cancer patients (Sotiriou *et al.* 2003). The significance of the upregulation of CKS2 by HRG reported here is unclear at this stage and requires further investigation.

GADD45A was transiently upregulated by both EGF and HRG in C3.6 cells, and by EGF in HB4a cells, and has previously been shown to interact with a number of cell cycle regulating proteins, including PCNA, p21 and CDK1-CyclinB complex (Hildesheim & Fornace, Jr. 2002). GADD45B, another family member thought to be involved in the activation of the S and G2/M checkpoints (Vairapandi *et al.* 2002), was upregulated in both cell lines by EGF. E2F4, the most abundant member of the E2F family, was transiently upregulated in C3.6 cells following HRG stimulation despite reports showing that it is constitutively expressed throughout the cell cycle and in quiescent cells (Beijersbergen *et al.* 1994,Ginsberg *et al.* 1994). These reports contradict the present results and highlight the need for further research into the regulation of E2F family members.

It is important to consider that 4h, the first time point in the series used here, may be a time point that is too late to notice differences in the expression of immediate-early genes as their mRNAs could have returned to basal levels as cells progress through the cell cycle. This point is worth mentioning in relation to the expression of the cyclins. Previous microarray studies performed in this laboratory using the same cell system (White *et al.* 2004) found upregulation of cyclin-D2

(CCND2). This was not the case here, where none of the cyclin genes represented on the array (CCNA1, CCNA2, CCND2, CCND3, CCNE1 and CCNG2) showed significant changes in gene expression between cell lines or after growth factor treatment, despite the observation of increased mitogenic induction of cyclins D1 and E1 at the protein level in the ErbB2 overexpressing cells (Timms *et al.* 2002). Further studies are thus required to establish how indeed cyclin expression is regulated.

Among other genes involved in regulating cellular proliferation which were identified were IL6ST and prohibitin (PHB). IL6ST, also known as gp130, was downregulated in C3.6. It is a signal-transducing subunit shared by the receptors for the interleukin (IL)-6 family of cytokines, which includes IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1, and cardiotrophin-like cytokine. These cytokines have diverse functions and have been implicated in inflammatory and immune responses, heart development and fertility (Muller-Newen 2003). Signal transduction through IL6ST is mediated by two pathways: the JAK-STAT pathway and the Ras mitogen-activated protein kinase pathway. Upon ligand binding, gp130 becomes phosphorylated by JAK, resulting in the recruitment and activation of SHP2, which in turn can activate the Ras-ERK MAPK pathway, and of STAT3, which then forms homo- or hetero-dimers that translocate to the nucleus to activate the transcription of target genes (Stahl *et al.* 1995). These findings are interesting because they provide another example of downregulation of a gene involved in cytokine signalling through the JAK-STAT pathway, in addition to the IFN-related genes discussed below. Prohibitin, on the other hand, was found to be constitutively up-regulated in C3.6 cells and also showed upregulation after EGF and HRG treatment. Paradoxically, prohibitin is thought to be a tumour suppressor because it negatively regulates cell cycle progression by binding to pRb and repressing E2F transcriptional activity (Wang *et al.* 1999). Downregulation of prohibitin has been reported in gastric tumours (Jang *et al.* 2004), and there are reports of prohibitin mutations in certain sporadic breast cancers (Sato *et al.* 1993). Thus, the findings reported here require further investigation to determine the exact mechanism of prohibitin regulation and function in breast cancer development.

3.7.4 Genes involved in protein targeting and trafficking

RAB7 was downregulated in C3.6 compared to HB4a cells. Rab GTPases are critical regulators of endocytic transport, and are involved in regulating vesicular traffic. RAB7 is thought to regulate traffic of molecules destined for degradation from early to late endosomes (Feng *et al.* 1995,Stein *et al.* 2003). Until recently, no direct evidence for a role of RAB7 in cancer has been established, although one report suggests that RAB7 acts as a pro-apoptotic factor following growth factor withdrawal through the downregulation of nutrient transporters (Edinger *et al.* 2003). These results connect RAB7 with apoptosis which is often suppressed in tumour cells. Interestingly, RAB7 expression was found to increase late endosomal transport of EGFR (Dong *et al.* 2004). As EGFR is known to be endocytosed as a mechanism of negative regulation, it is possible that RAB7 downregulation due to ErbB2 overexpression in C3.6 cells may decrease the endocytosis rate of receptors from the plasma membrane and thus prolong signalling in these cells. Together, the observed downregulation of protein phosphatases and of RAB7 supports the role of ErbB2 in malignant transformation through the inhibition of negative regulatory mechanisms that are functional in normal cells. Other RAB family members, RAB1B and RAB27A, were also present in the "ErbB2 only" upregulated genes but were not significantly different at T0. RAB27 is membrane bound and thought to be involved in exocytosis (Stein *et al.* 2003,Izumi *et al.* 2003) while RAB1B regulates vesicular trafficking between the endoplasmic reticulum and the Golgi complex (Plutner *et al.* 1991). SCAMP2 was also upregulated in C3.6 cells. This gene encodes for an integral membrane protein with four transmembrane spans, and it is likely to participate in late-stage vesicle exocytosis (Liu *et al.* 2002b). There have been suggestions that tyrosine phosphorylation of other SCAMPs, but not SCAMP2, may functionally participate in the internalization of EGFR (Wu & Castle 1998).

Copine III (CPNE3) was present in the "ErbB2 only" gene list, reflecting its upregulation in C3.6 cells. The cellular function of this gene is not fully understood, though it is thought to be involved in membrane trafficking by virtue of its lipid and Ca^{2+}-binding C2 domains (Creutz *et al.* 1998). Note that the other CPNE3 clone represented on the array was among the "HRG only" genes, having failed statistical tests when comparisons between the two cell lines were made (see Figure 3.8). This discrepancy of results makes it difficult to interpret such findings and highlights the

need for other methods for validation of the microarray data. However, this finding is supported by the fact that CPNE3 was also found to be upregulated in C3.6 cells in the microarray analysis of White *et al* (White *et al.* 2004) and such changes in mRNA would have resulted in a similar pattern of protein expression when lysates of serum-starved and growth factor-stimulated HB4a and C3.6 cells were compared using two-dimensional difference gel electrophoresis (2D-DIGE) (Gharbi *et al.* 2002). In these expression profiling experiments, CPNE3 protein was overexpressed in C3.6 cells and was upregulated by HRG. Prior to work carried out in our laboratory, the overexpression of CPNE3 had not been reported in human cancers or related to ErbB-2 overexpression or growth factor signalling. Thus, this gene represents a potential novel potential target involved in ErbB2-mediated cellular transformation, and further studies into the mechanisms of CPNE3 action should be carried out. Interestingly, a number of genes identified here also showed altered expression patterns at the protein level (Gharbi *et al.* 2002,White *et al.* 2004,Chan *et al.* 2005). By correlating mRNA and protein expression changes it is possible to predict how the activity of a gene is regulated. These correlation studies are further discussed in Chapter 4 – Target Validation and Characterization.

CLTC (clathrin heavy chain 1), another gene involved in protein transport, was found to be constitutively upregulated in the C3.6 cells relative to HB4a cells (Table 3.2). Clathrin is a major protein component of the cytoplasmic face of cellular membranes, and forms coated vesicles and coated pits. Clathrin coated vesicles and pits are responsible for intracellular trafficking of receptors and endocytosis of a variety of macromolecules. The basic subunit of the clathrin coat is composed of three heavy chains and three light chains (Brodsky *et al.* 2001). It is now well established that clathrin mediates the endocytosis of activated EGFR, thereby regulating signalling through this receptor. A possible explanation for the upregulation of CLTC in C3.6 cells is that ErbB2 may modulate the rate of endocytosis and degradation of other ErbB family members, supporting the finding that basally the C3.6 cells express lower levels of EGFR than HB4a cells (Figure 3.1 and (Timms *et al.* 2002). Previous work in our laboratory showed that EGF treatment triggers EGFR internalisation and degradation with increased rates observed in the ErbB2 overexpressing cells, reflecting the decreased overall expression level in C3.6 cells (Severine Gharbi, Thesis, 2002). It is tempting to speculate that the differential

expression of CLTC is involved here, although a direct pathway from ErbB2 to CLTC has not been established.

A group of genes involved in the transport of proteins across the endoplasmic reticulum (ER) were also differentially expressed in C3.6 cells. Translocation across the ER membrane is a fundamental phase in the biogenesis of secretory and membrane proteins, and most of the components of the translocation machinery have been identified. Here, TRAM1 (translocation associated membrane protein 1) and SRP14 (signal recognition particle 14) were constitutively upregulated in C3.6 cells. SRP14 has a crucial role in targeting secretory proteins to the ER membrane (Terzi *et al.* 2004), while TRAM is responsible for the translocation of most secretory proteins across the ER membrane (Voigt *et al.* 1996). KDELR2 (KDEL receptor-2), which was consistently downregulated in C3.6 cells, is required for the retention of luminal endoplasmic reticulum proteins. KDEL receptor was found to play an important role in ER quality control, a mechanism by which the transport of misfolded or partially unassembled proteins to their final cellular destination is prevented by the retention of such proteins in the ER (Yamamoto *et al.* 2001). The role of ErbB2 overexpression in the translocation of proteins through the ER is a potentially interesting new aspect in signalling research and further studies are required to understand this effect.

Finally, both caveolin-1 and 2 (CAV1 and CAV2) were among the differentially expressed genes identified in this analysis. CAV1 was upregulated following EGF stimulation of both cell lines at T4 and T18, and CAV2 was transiently upregulated by HRG in C3.6 cells. CAV1 was originally isolated from plasma membrane invaginations called caveolae, which are specialized invaginations of the plasma membrane that have been proposed to play a role in diverse cellular processes such as endocytosis and signal transduction.(Rothberg *et al.* 1992). CAV1 plays an important structural role in forming caveolar invaginations on the plasma membrane (Fra *et al.* 1995), and it was shown to interact with a variety of lipids (e.g. cholesterol) and proteins (e.g. receptor and non-receptor tyrosine kinases), suggesting that it may function as a scaffold protein that sequesters and organizes these molecules into multimeric signalling complexes (Liu *et al.* 2002c). CAV2 is colocalized and co expressed with CAV1 and both genes have been mapped to a common locus in chromosome 7q31.1, consistent with the functional relationship of these two proteins in the formation of hetero-dimers (Fra *et al.* 2000). CAV1 has

been shown to interact with EGFR and to negatively regulate its kinase activity (Couet *et al.* 1997). CAV1 expression is undetectable or very low in many human tumour cell lines (Koleske *et al.* 1995,Lee *et al.* 1998,Bender *et al.* 2000,Bagnoli *et al.* 2000) and mitogenic signalling pathways acting through the Ras-ERK1/2 cascade have been shown to negatively regulate transcription of CAV1 and CAV2 (Engelman *et al.* 1999). Thus, the finding here that CAV1 and CAV2 are upregulated by the growth factors used here is surprising and further studies are required to understand the regulation of CAVs in response to growth factors in these cells.

3.7.5 Cytoskeleton & ECM-related genes

It is well established that tumour forming cells undergo morphological transformation, normally accompanied by changes in the expression of genes involved in actin organization as well as essential structural genes. Furthermore, growth factors are known to be important factors in the regulation of actin and the cytoskeleton. Genes differentially expressed in this analysis that are involved in cytoskeleton and/or ECM signalling have been mentioned above. These include ZYX, VCL, TAGLN and CNN3 (changing in response to all conditions); TGM2, TUBB2, TUBA1, VIL2 and ITGA3 (changing in response to EGF and HRG); and LAMA3, ACTB, CDH3, CTNNAL1 and VASP (responsive to EGF only); VIM, FBLN2, KRT13, KRT15 and GPC1 (responsive to ErbB2 and HRG); and annexins 1 and 2 (differentially regulated between HB4a and C3.6 cells at T0 and upregulated by EGF), which bind F-actin (Gerke & Moss 2002), and may play a role in regulating membrane-cytoskeleton dynamics (Gerke & Moss 1997).

A number of integrins, namely integrin-α2 (ITGA2), integrin-α3 (ITGA3), integrin-β4 (ITGB4) and integrin-β4 binding protein (ITGB4BP), were identified in this analysis. They were present in the ErbB2 gene list and their expression could be induced by both growth factors, although the effect of ErbB2 was gene specific (Figure 3.27). Integrins are transmembrane heterodimeric molecules composed of α and β subunits and provide a link between the extracellular matrix (ECM) and the cytoskeleton, regulating important processes such as adhesion, cell proliferation, and survival. They interact with the actin cytoskeleton through the association of their cytoplasmic tails with actin-binding proteins such as α-actinin, filamin, vinculin and talin (Burridge & Chrzanowska-Wodnicka 1996,Yamada & Geiger 1997,Geiger *et al.*

2001). It has been previously shown that integrins can induce EGFR tyrosine phosphorylation in the absence of EGFR ligands, leading to SHC tyrosine phosphorylation and activation of the ERK/MAP kinase pathway (Moro *et al.* 1998). Here, the differential induction of the integrin subunits α2, α3 and β4 by EGF and HRG in the HB4a and C3.6 cells may promote altered adhesive properties between these two cell lines.

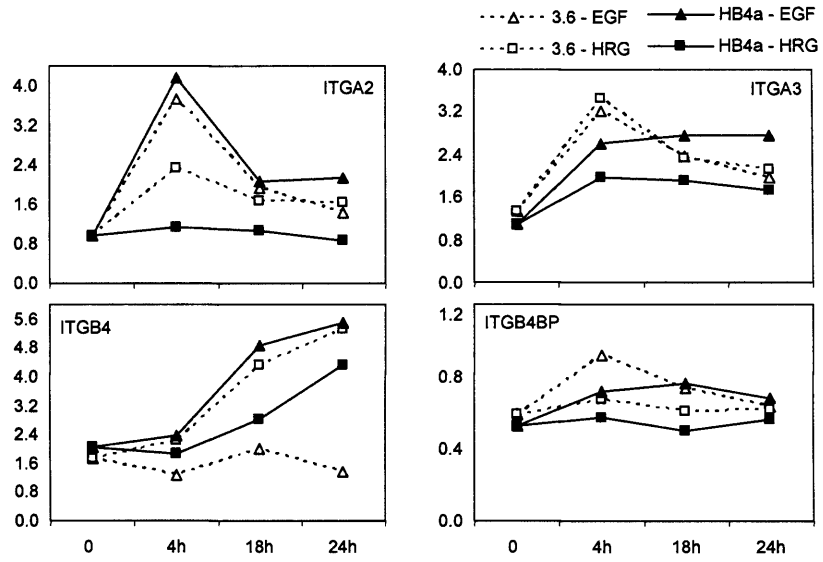

Figure 3.27: Expression levels of integrin family genes. Genes from the integrin family found to be significantly changing by SAM. The differences between HB4a and C3.6 cells are due to variations in growth factor responsiveness rather than due to ErbB2 itself.

Mucin-1 (MUC1) levels were lower in the C3.6 cells and it was also downregulated by EGF treatment. MUC1 encodes a type-I transmembrane glycoprotein expressed on the surface of most epithelia, including mammary gland, prostate and lung, and is thought to modulate cell-cell and cell-matrix interactions (Brayman *et al.* 2004). MUC1 has been reported to be overexpressed in breast carcinomas and to contribute to metastasis through its anti-adhesion properties (Hilkens *et al.* 1995), a finding that contradicts the results presented here. MUC1 may also play a role in cell signalling, since its cytoplasmic tail has been shown to associate with signalling molecules such as Grb2 and Sos (Pandey *et al.* 1995) and β-

catenin (Yamamoto *et al.* 1997). The MUC1 cytoplasmic tail has also been shown to directly bind EGFR and to be phosphorylated by EGFR following EGF stimulation, and transgenic mice overexpressing MUC1 in mammary glands show enhanced activation of MAPK (Schroeder *et al.* 2001). These findings suggest that MUC1 may regulate cell growth and differentiation via the Grb2-Sos-Ras-MEK-ERK2 signalling pathway. On the other hand, recent reports show that ErbB2 homodimerization can inhibit MUC1 expression at the transcriptional level (Canbay 2003), and transfection studies showed that co-transfection of ErbB2 inhibited transcription of a reporter gene driven by the MUC1 promoter (Scibetta *et al.* 2001), supporting the data presented here. Despite these data, the role of MUC1 in cellular physiology and malignancy is still unclear and further studies are required to clarify its relevance in breast cancer.

L-plastin (LCP1), a member of the plastin family of actin-bundling proteins, was found to be significantly upregulated in C3.6 cells. L-plastin appears to contribute to the regulation of cell adhesion and motility (Jones *et al.* 1998). Although L-plastin expression was previously thought to be specific to heamopoietic cell lineages, it has been found in many types of malignant human cells of non-heamopoietic origin, suggesting that its expression is induced during tumorigenesis in solid tissues (Lin *et al.* 1988a,Park *et al.* 1994). In a study of 50 human tumour cell lines derived from non-heamopoietic origin, 63% expressed L-plastin, with a particularly high frequency of L-plastin expression seen in ovarian and breast tumour cell lines (Lin *et al.* 1993). Furthermore, the expression of L-plastin has been correlated with cancer invasion and metastasis, suggesting that it could be used as a clinical marker of metastatic cancer (Otsuka *et al.* 2001). Importantly, the functional involvement of L-plastin in invasion and metastasis was shown in an antisense study in PC3 prostate carcinoma cells, where cells transfected with an antisense L-plastin gene were shown to display suppression of *in vivo* invasion in nude mice (Zheng *et al.* 1999). Despite this, the mechanism of action of L-plastin remains unclear and further studies are required to relate the role of its actin-binding properties with the up-regulation of cell proliferation and motility.

A number of genes involved in extracellular matrix (ECM) remodelling were analysed to see if they may have roles in ErbB2-mediated metastasis. St14 was consistently upregulated in C3.6 cells. This gene, also known as membrane-type serine protease-1 (MT-SP1), was originally isolated as a novel protease expressed in

human breast carcinoma cells (Shi *et al.* 1993) and has been proposed to play a role in breast cancer invasion and metastasis through its effects on post-translational activation and degradation of ECM components (Benaud *et al.* 2002). In addition, St14 functions as an activator of other proteases and latent growth factors, such as the urokinase plasminogen activator (uPA) and the hepatocyte growth factor (HGF), both of which have been implicated in the growth and motility of cancer cells (Oberst *et al.* 2003). Thus, ErbB2-dependent downregulation of St14 may promote tumour progression by acting as an upstream activator of HGF and uPA or by directly remodelling the ECM. PLAUR, the uPA receptor, was among the 775 genes identified in this analysis, but its expression levels only changed in response to EGF.

MMPs are also involved in the degradation of ECM components and are thought to play a crucial role in tumour invasion (Folgueras *et al.* 2004). Specifically, MMP3 and MMP9 have been reported to be involved in breast tumourigenesis (Sternlicht *et al.* 1999,Ranuncolo *et al.* 2003). Of the 19 MMP clones present of the microarrays, representing 11 unique family members, none showed differences in expression levels in any of the conditions studied, despite it being shown that a number of agents, including growth factors, cytokines and oncogene products, can induce the expression of MMPs (Folgueras *et al.* 2004) and promoter studies having revealed the existence of AP-1 binding sites in the promoter regions of many MMP genes (Pendas *et al.* 1997). One possible explanation for these findings is that MMP overexpression is associated with more advanced tumour stages, which the HMLEC model used here does not represent. Furthermore, there are other levels of regulation of MMP activity. For example, MMPs are synthesized as inactive zymogens, and their activation by the removal of the regulatory domain provides another step in the regulation of MMP activity (Nagase 1997). Additionally, a number of endogenous inhibitors of MMPs exist that control their activity, including the tissue inhibitors of metalloproteinases (TIMPs) (Brew *et al.* 2000). Indeed, TIMP overexpression suppresses primary tumour growth and metastasis (DeClerck *et al.* 1992,Bian *et al.* 1996,Nagase 1997,Wang *et al.* 1997,Yamauchi *et al.* 2001), suggesting that inhibition of MMPs may have therapeutic potential. Both TIMP1 and TIMP3 were identified in this microarray analysis; TIMP1 was induced by EGF more highly in HB4a cells compared to C3.6, and TIMP3 was upregulated by HRG only in HB4a cells, and returned to basal expression levels by T24. These results support the hypothesis that, in ErbB2-related breast cancers, MMPs are indirectly de-regulated

through TIMP expression. The findings shown here in combination with the findings that i) ErbB2 mRNA levels correlate with TIMP1 mRNA levels in breast carcinomas (Nakopoulou *et al.* 2002), ii) ErbB2 can induce the transcription of MMP1 (Bosc *et al.* 2001), and iii) MMPs, particularly MMP9, can be induced by EGF and HRG (O-charoenrat *et al.* 1999), highlight the need for further studies of the role of ErbB2 and ErbB ligands in the regulation of MMPs and TIMPs.

Finally, the clone 324061_A was found to represent fibronectin. Fibronectin (FN1), a major ECM component required for integrin-mediated signalling (Ruoslahti 1999), has been reported to be downregulated in C3.6 cells in two other microarray studies (Mackay *et al.* 2003,White *et al.* 2004). Although FN1 was generally expressed at lower levels in C3.6 cells, these expression changes were not found to be significant by SAM. Fibronectin did however show a very large and significant upregulation in response to HRG stimulation in both cell lines. FN1 was previously found to be downregulated in C3.6 cells, and these cells were found to be significantly less adhesive that HB4a cells (White *et al.* 2004). However, C3.6 adhesion was increased on FN-coated plastic, consistent with a role for FN1 in regulating the cellular adhesive properties of the C3.6 cells. HRG stimulation was also shown to increase the adhesive properties of C3.6 cells (White *et al.* 2004), and the upregulation of FN1 observed here could therefore represent an explanation to the findings by White *et al.*

Further studies are required to elucidate the interplay between ErbB2 signalling and adhesion. Nonetheless, these data suggest that ErbB2 overexpression results in an altered cellular phenotype, manifested as a change in cellular morphology and possibly in altered cell-cell and cell-ECM interactions. These changes in the expression of structural genes could account, at least in part, for the different morphologies displayed by the two cell types (Figure 3.1-b) and for the increased invasive potential of ErbB2-overexpressing cells (Xu *et al.* 1997).

3.7.6 Energy pathways, oxidative stress & metabolic enzymes

Many of the differentially expressed genes were found to be involved in cellular metabolism. ALDH1A3 (aldehyde dehydrogenase 3) was the gene that displayed the highest upregulation in the ErbB2-overexpressing cells. The superfamily of ALDHs consists of 20 gene families with at least 172 distinct genes

and are involved in the metabolism of exogenous or endogenous aldehydes generated by alcohol metabolism and lipid peroxidation (Sophos & Vasiliou 2003). AKR1B1 (aldose reductase 1B1) was also upregulated in C3.6 cells and participates in glucose metabolism and osmoregulation, as well as catalyzing the reduction of a number of aldehydes (Bohren *et al.* 1989). Overexpression of aldose reductase in liver cells has been previously shown to induce resistance to the chemotherapy drug daunorubicin (Lee *et al.* 2001). While differential expression of metabolic enzymes has been reported in breast cancer (Hennipman *et al.* 1988,Mathupala *et al.* 1997), it is important to note that the observed increased expression of metabolic enzymes in the C3.6 cells could occur as a consequence of the enhanced proliferation of these cells in response to ErbB-2 overexpression and the increased energy requirements associated with this. This possibility is reinforced by the finding that a number of genes involved in ATP synthesis were also constitutively upregulated in C3.6 cells, namely ATP5G1 (all three represented clones), ATP5G3 (two represented clones), ATP5L and ATP6V1F.

Reactive oxygen species (ROS) were originally thought to only be released by phagocytic cells during their role in cell defence. However, they are now believed to play an important role in activating signalling pathways such as those including MAPKs, Akt, PKC and phospholipase C-γ thus regulating cell proliferation and survival (Kamata & Hirata 1999). ROS can interact with and damage various cellular components, such as DNA, proteins, carbohydrates or lipids and increased ROS production has been implicated in the development of many diseases, including cancer (Behrend *et al.* 2003). One of the most important enzymes in the generation of ROS is NADPH oxidase. The catalytic core of the oxidase is the membrane electron transporting component cytochrome b_{558}, a heterodimer composed of CYBA (p22-*phox*) and CYBB (gp90-*phox*). Other components of the NADPH oxidase enzyme reside in the cytoplasm, but are recruited to form an activated membrane-associated NADPH oxidase complex (Hancock *et al.* 2001). Here, CYBA was found to be constitutively downregulated in C3.6 cells in agreement with the previous microarray analysis performed in our lab (White *et al.* 2004). Although there are no reports to date connecting CYBA and ErbB2 expression levels, ROS are known to play a role in oncogenic transformation (Behrend *et al.* 2003), and indeed in breast cancer (Brown & Bicknell 2001). Specifically, NADPH oxidase-dependent superoxide production has been linked with cancer progression (Brar *et al.*

2002,Perner *et al.* 2003). In both studies, CYBA expression was found to be necessary for O_2^- production in tumour cells. The fact that CYBA is under-expressed in the C3.6 cell goes against the hypothesis that breast cancer cells show increased signalling due to high production of ROS. The correlations in expression and hence the possible relationship between CYBA and ErbB2 presented here represents an interesting testable hypothesis that will require further investigation.

Another source of ROS is the mitochondrial respiratory chain. Cytochrome c oxidase (COX) is the terminal member of the electron transport chains of mitochondria, acting as a proton pump. It catalyses the electron transfer of reduced cytochrome c to oxygen, and is thought to be involved in apoptosis (Kadenbach *et al.* 2004). COX is composed of 13 subunits, three of which are encoded by mitochondrial DNA (subunits I, II and III), whereas the remaining 10 are encoded by the nuclear genome and occur as tissue-specific isoforms (Ludwig *et al.* 2001). Both COX6C and COX8 were upregulated in C3.6 cells. The function of these subunits is still unknown. However, recent reports have shown that there is a higher level of nuclear encoded COX subunits in tumour-derived cell lines than mitochondrial subunits (Krieg *et al.* 2004), and COX6C has been shown to be upregulated in prostate cancer cells and tissues (Wang *et al.* 1996,Herrmann *et al.* 2003). Little is known about COX8 and its role in transformation.

As mentioned above, the formation of excessive amounts of ROS can be toxic to cells. Thus, cells possess a tightly regulated system to metabolise and scavenge ROS to maintain the cellular redox environment in a reduced state (Figure 3.28). It is therefore possible that de-regulation of the redox status in cancer cells is an indirect consequence of malfunction of these ROS regulatory systems, where inefficient detoxification of various carcinogens could lead to genetic damage and increased cancer risk.

The de-regulation of redox regulatory mechanisms in C3.6 cells has been previously established in our lab through parallel proteomics experiments using 2D-DIGE technology (Gharbi *et al.* 2002,Chan *et al.* 2005). Superoxide Dismutase (SOD) is responsible for the conversion of O_2^- into H_2O_2, which is then degraded to H_2O by several cellular enzymes (Miller 2004). While MnSOD was found to be downregulated in C3.6 cells in the proteomics experiments, none of the members of the SOD family (Mn-SOD, Cu,Zn-SOD and EC-SOD) showed statistically significant changes in gene expression between HB4a and C3.6 cells. This suggests

that whilst the redox status may indeed be de-regulated in ErbB2-related cancers, this occurs at the level of protein regulation rather than gene transcription. Peroxiredoxins (PRDX) are another family of antioxidant proteins that are closely associated with thioredoxin (TRX)-dependent reactions (Figure 3.28), being also known as TRX peroxidases (Wood *et al.* 2003). At least six PRDXs have been identified in mammalian cells. PRDX3 and PRDX5 were downregulated in C3.6 cells in 2D-DIGE proteomics experiments, and these results were further confirmed by western blotting (Chan *et al.* 2005). Although PRDXs were not changing in the microarray experiments presented here when compared between HB4a and C3.6 cells, PRDX1 was found to be significantly upregulated by EGF, and PRDX5 was downregulated by both EGF and HRG. The relevance of these findings is still unclear, and further studies are underway to determine the role of ErbB2 in regulating PRDX protein expression and activity and in regulating the redox status of cells.

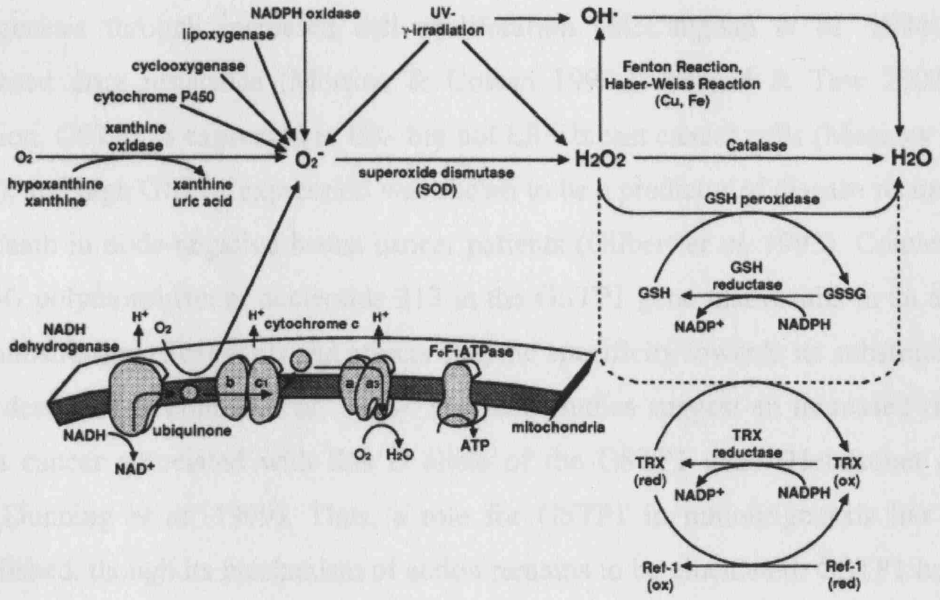

Figure 3.28: Regulation of cellular redox status (from: (Kamata & Hirata 1999)). Reactive oxygen species (ROS) are generated in cells by several pathways, including NADPH oxidase and cytochrome c, which have been identified in this analysis. Superoxide dismusate (SOD) converts O2- into H2O2, which is then degraded to H2O by glutathione (GSH) peroxidase and catalase. Two members of the GST family, enzymes responsible for conjugating GSH to various electrophilic compounds (not shown in this figure), were also found to be differentially expressed. Thioredoxin (TRX) also has H2O2 reducing activity, as well as a function in refolding oxidized proteins.

Glutathione (GSH) is another major cellular reductant. H_2O_2 and other peroxides are eliminated by a GSH peroxidase-catalysed reaction, using GSH as a substrate. As a result of this reaction, oxidised GSH (GSSG) is formed, which is then reduced back to GSH by GSH reductase (GR) (Figure 3.28) (Townsend *et al.* 2003). Glutathione S-transferases (GSTs) are a family of enzymes that function to protect cellular molecules from irreversible damage by ROS by catalysing the conjugation of GSH to a number of electrophilic compounds. Human GSTs are divided into two distinct super-families: the membrane bound microsomal and the cytosolic family members. The cytosolic members are further divided into six classes: Alpha (GSTA1-2), Mu (GSTM1-5), Omega (GSTO1), Pi (GSTP1), Theta (GSTT1-2) and Zeta (GSTZ1) (Townsend *et al.* 2003). Here, GSTP1 expression was found to be constitutively lower in C3.6 cells compared to HB4a and these results were confirmed at the protein level (Chan *et al.* 2005). Paradoxically, high GSTP1 expression levels have been observed in tumours from a number of human tissues, with GSTP1 levels correlating with the degree of de-differentiation and malignancy (Tsuchida & Sato 1992,Xia *et al.* 1996). GSTP1 expression has been implicated in oncogenesis through increased cell proliferation (McCaughan *et al.* 1994) and increased drug resistance (Morrow & Cowan 1990,Townsend & Tew 2003). In addition, GSTP1 is expressed in ER- but not ER+ breast cancer cells (Moscow *et al.* 1988), and high GSTP1 expression was shown to be a predictor of disease recurrence and death in node-negative breast cancer patients (Gilbert *et al.* 1993). Conversely, an *A-G* polymorphism at nucleotide 313 in the GSTP1 gene that results in an amino acid substitution (Ile104val) and affects enzyme specificity towards its substrates has been described (Zimniak *et al.* 1994). Previous studies suggest an increased risk of breast cancer associated with this *G* allele of the GSTP1 gene (Helzlsouer *et al.* 1998,Dunning *et al.* 1999). Thus, a role for GSTP1 in tumourigenesis has been established, though its mechanism of action remains to be elucidated. GSTP1 has not been previously linked to ErbB2 overexpressing breast cancers and therefore the results discussed here offer an alternative hypothesis for the involvement of this gene in breast cancer. The membrane bound microsomal GST, or MGST1, was upregulated in C3.6 cells but did not display growth factor responsiveness. Although this gene is induced during oxidative stress (Kelner *et al.* 2000), it is more often activated by post-translational modifications such as proteolysis, ligand-binding and thiol-disulfide interchange (Rinaldi *et al.* 2004). The role of MGST1 in cancer is

currently less clear and further studies are required to characterize the involvement of this gene in ErbB2-related cancer.

3.7.7 Genes involved in ubiquitin & ubiquitin-like protein modification

Protein activity can be regulated by a variety of mechanisms. These include control of expression levels through regulated gene expression, translation, degradation and localization or through constitutive or reversible post-translational modifications. One post-translational modification which can affect protein turnover is ubiquitination, the enzymatically catalyzed formation of an isopeptide bond between a target protein and polymeric ubiquitin. Typically, tagging a protein with ubiquitin chains usually leads to its complete degradation by the 26S proteasome. However, ubiquitination can also control activation of proteins via limited proteolysis and appears to play a role in receptor-mediated endocytosis. The conjugation of ubiquitin to a target protein requires the activity of the ubiquitin activating enzyme (E1) which hydrolyses ATP to form a thiolester-linked complex with ubiquitin. Ubiquitin is then transferred to one of several ubiquitin-conjugating enzymes (E2s) via a similar thiolester linkage. Finally, ubiquitin-protein conjugates are generated by specific ubiquitin ligases (E3s) that interact directly with the protein substrate and transfer ubiquitin from E2s. Usually, several ubiquitin molecules are conjugated to a substrate in the form of a poly-ubiquitin chain, although this is not always the case (Scheffner *et al.* 1995). There are also several ubiquitin-related proteins that have been described which fall into two main groups: ubiquitin-like modifiers (UBLs), which function in a similar manner to ubiquitin by attaching to target proteins, and ubiquitin-domain proteins (UDPs), which bear protein domains that are related to ubiquitin but do not conjugate to other proteins (Jentsch & Pyrowolakis 2000).

A number of genes involved in the ubiquitin cycle were identified in this analysis. Ubiquitin itself (UBB/UBC) was present in the "ErbB2 & HRG" gene list, as it was found to be significantly downregulated by HRG only in the C3.6 cells. The ubiquitin conjugating enzymes UBE2D2 and UBEN were upregulated by EGF but showed no difference in expression between the two cell lines, whilst UBE2L6 was constitutively downregulated in C3.6 cells. UBE2L6 has been shown to bind the E3 enzyme E6AP, which implicates this gene in the ubiquitination and downregulation

of the tumour suppressor p53 (Kumar *et al.* 1997). Other downregulated genes were USP14, a ubiquitin-specific protease involved in protein deubiquitination (Borodovsky *et al.* 2001), RPN1, a non-ATPase subunit of the 19S proteasome regulatory particle that may act as a receptor for binding ubiquitin-like proteins (Elsasser *et al.* 2002) and TRIP12, a protein thought to have ubiquitin protein ligase activity due to its significant sequence similarity to the E3 enzyme E6AP (Lee *et al.* 1995b).

Other genes involved in protein modification were constitutively downregulated in C3.6 cells, and did not show any responsiveness to growth factors. G1P2, or ISG15, was the most downregulated gene in C3.6 cells. This gene is highly inducible by IFNs and is thought to play a role in protein modification in a similar fashion to ubiquitin in a process known as ISGylation (Haas *et al.* 1987,Loeb & Haas 1992). However, unlike ubiquitin, only an E1 conjugating enzyme for ISG15 has been identified (UBE1L), indicating a clear difference between ISGylation and the ubiquitination pathway, although UBE2L6 (discussed above), may act as an E2 enzyme for ISG15 (Zhao *et al.* 2004), and is itself induced by IFNα (Nyman *et al.* 2000). Little is known about the effects of ISG15, though it is believed ISGylation has an important role in mediating some of the diverse biological effects of interferon. Targets of ISG15 modification described to date include Serpin2a, ERK1/2, phospholipase Cγ1, STAT1 and Jak1, all of which are molecules involved in critical signal transduction processes (Malakhov *et al.* 2003,Ritchie & Zhang 2004). However, the fate of the ISG15-modified proteins has not yet been established. There have been reports linking the proteasome with ISGylation; one group showed that treatment with proteasome inhibitors decreased the amount of ISG15 mRNA and protein (Li & Hassel 2001) and addition of proteasome inhibitor to interferon pre-treated cells enhanced protein ISGylation (Liu *et al.* 2003), whereas another group showed that proteasome inhibitors did not affect the level of protein ISGylation (Malakhov *et al.* 2003). It is possible that long treatment with proteasome inhibitors may increase the amount of ISG15 conjugates as a consequence of increased stress to cells. It therefore remains to be established whether there is indeed a link between ISG15 conjugation and the proteasome. The involvement of ISG15 in ErbB2-mediated transformation is further discussed in the context of interferon signalling in Chapter 5.

Another ubiquitin-like gene downregulated in C3.6 cells was UBL1, also known as SUMO1 (small ubiquitin-related modifier). Both UBL1 clones present in the array were downregulated and present in the "ErbB2 only" list. Similar to ubiquitin and ISG15, UBL1 conjugates to acceptor proteins in a reaction mediated by the E1 heterodimer AOS1/UBA2 and the E2 conjugating enzyme UBC9. Substrates can be modified by several UBL1 molecules at distinct sites, but no poly-UBL1 chains seem to be formed. A number of UBL1 targets have been identified, many of which are nuclear transcription factors or transcription-associated proteins such as Sp3, c-Jun, c-Myb, AP2, p300 and HDAC1. UBL1 conjugation can have either a stimulatory or an inhibitory effect on gene expression, but most studies have shown UBL1 modification to correlate with downregulation of their transcriptional activation potency (Verger *et al.* 2003,Girdwood *et al.* 2004). The role of UBL1 is not limited to transcription. The first UBL1 substrate identified was RanGAP1, a GTPase-activating protein of the Ras-related GTPase Ran, which functions to control nucleo-cytoplasmic transport (Matunis *et al.* 1996). Only the UBL1-modified RanGAP1 was found to be associated with RanBP2, a protein at the cytoplasmic face of the nuclear pore complex that serves as a docking site for import complexes. This suggests that UBL1 may function to target RanGAP1 to the nuclear pore and stabilize the association between RanGAP1 with RanBP2 (Mahajan *et al.* 1997). In fact, UBL1 is emerging as a versatile modifier of a large number of proteins in many different pathways, and the consequences of this modification seem to be as diverse as its targets, which include proteins involved in DNA replication and repair (Rad52, PDNA, topoisomerases), mitosis (Cdc3, Cdc11, Sep7) and signal transduction (p53, E2A, TNFα receptor, androgen and glucocorticoid receptors) (Melchior 2000). Furthermore, UBL1 has been shown to compete with the ubiquitin pathway, acting as an antagonist of ubiquitin by stabilizing proteins through modification of the same residues that are ubiquitinated (Desterro *et al.* 1998,Hoege *et al.* 2002).

Collectively, these results suggest that protein modification by ubiquitin and ubiquitin-like proteins is a key factor in ErbB2-dependent signalling and transformation.

3.7.8 *Interferon-stimulated genes*

A significant number of genes involved in the interferon (IFN) signalling pathway were downregulated in the ErbB2-overexpressing cells, although there was little evidence of growth factor responsiveness (Figure 3.29). Indeed, of the 48 IFN-related genes represented on the array, 28 showed differential regulation in C3.6 cells compared to HB4a cells, 15 of which were differentially expressed at T0 when SAM analysis was carried out only on the IFN-related genes. IFNs are becoming increasingly recognized as important negative regulators of cell growth (Sangfelt *et al.* 2000) and therefore downregulation of the IFN signalling in ErbB2 overexpressing cells may be one mechanism by which these cells have an increased proliferative potential. IFN-dependent cellular effects are mediated by transcriptional induction of responsive genes, collectively referred to as IFN-stimulated genes (ISGs). As mentioned above, G1P2 (ISG15) was highly downregulated in C3.6 cells. In addition, and perhaps most importantly, ISGF3G, or p48, was downregulated in the ErbB2-overexpressing cells. ISGF3G, together with signal transducer and activator of transcription 1 (STAT1) and STAT2, forms the ISGF3 transcription complex (Stark *et al.* 1998). This complex is responsible for the transcription of IFNα/β-inducible genes, including IFNβ itself (Nakaya *et al.* 2001) and other genes identified here, such as OAS1, SP110, IFITM1, IFITM2 and indeed G1P2. Thus, downregulation of p48 in C3.6 cells may be the cause of lower transcription of other ISGs. Clustering of microarray data generated from the analysis of 8,000 genes in the NCI60 cancer cell lines resulted in the formation of a functional cluster showing distinct patterns of gene expression where all genes are known to be regulated by IFNs, suggesting that these cancer cell lines show variations in activity of the IFN response pathway (Ross *et al.* 2000). Furthermore, clustering of breast tumours could classify a subset of tumours based on the expression levels of IFN-regulated genes (Perou *et al.* 1999). These findings suggest that ISGs may indeed play a role in breast cancer development. However, the mechanisms by which IFN signalling may contribute to cancer development are not clear, and IFN signalling has not yet been associated with ErbB2 overexpression. The hypothesis presented here is that a cross-talk between ErbB and IFN signal transduction pathways exists. Further analysis and discussion on the IFN pathway will be presented in Chapter 5.

Interestingly, STAT1 was not found to be differentially regulated between HB4a and C3.6 cells in the microarray experiments, but was shown to be significantly upregulated by HRG. The microarray results obtained for STAT1 were validated by real time PCR (Chapter 4), and its role in the potential cross-talk between the ErbB and IFN-signalling pathways is further discussed in Chapter 5.

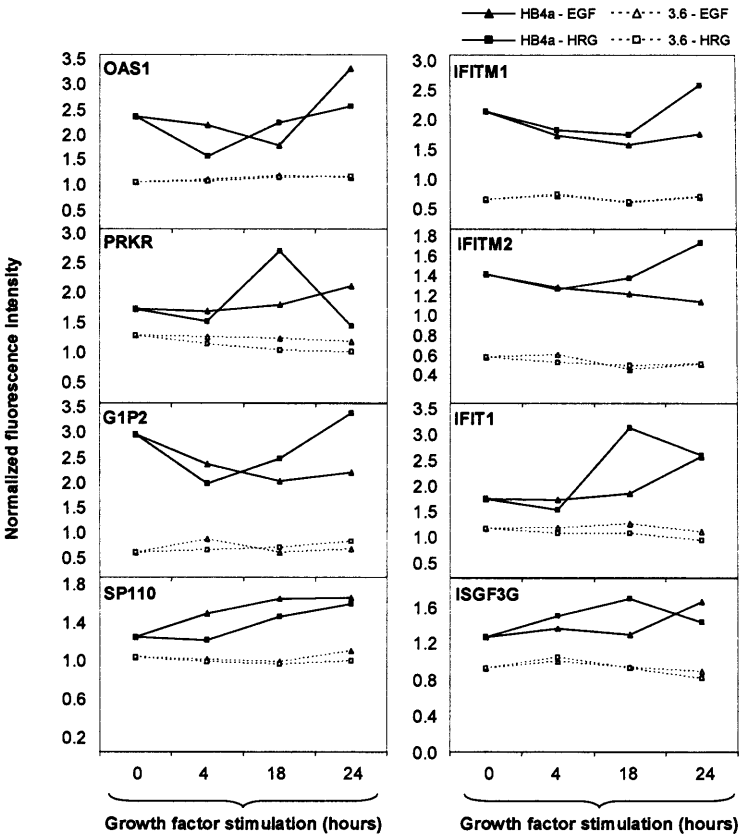

Figure 3.29: Downregulation of IFN-stimulated genes in the C3.6 cell line relative to HB4a. Normalized fluorescence intensities were plotted in Excel and represent the average ratio to the common reference sample. Genes were found to the statistically significant in at least one time point using SAM to compare C3.6 against HB4a cells.

3.7.9 S100 protein family

Five members of the S100 family of calcium-binding proteins were identified in this analysis (Figure 3.30). This family of proteins was originally characterized as a group of abundant low-molecular weight acidic proteins that are highly enriched in brain tissue, and today at least 19 family members have been described (Schafer & Heizmann 1996). Some S100 members are secreted into the extracellular space and

can act as chemo-attractants for leukocytes, modulate cell proliferation, or regulate macrophage activation. However, it is not yet clear how S100 proteins are secreted from cells or whether specific cell-surface receptors mediate their effects. S100 proteins have no known enzymatic activity and exert their intracellular effects by interacting with and altering the binding potential or availability of target proteins. Although in some cases different S100 proteins share their target proteins, in most cases a high degree of target specificity has been described, suggesting that individual S100 members might be implicated in the regulation of specific activities. Intracellular S100 proteins have been reported to exert pleiotropic effects, and these can be divided into several groups (Donato 1999). First, S100 proteins can regulate phosphorylation mediated by protein kinase C and thereby can modify the response mediated by this signal transduction pathway (Baudier *et al.* 1992). Second, they can regulate energy metabolism by modulating the activity of several target enzymes, such as fructose-1,6-bisphosphate aldolase and glycogen phosphorylase (Zimmer & Van Eldik 1986,Zimmer & Dubuisson 1993). Thirdly, S100 proteins can regulate cell shape by influencing the polymerization state of all three major constituents of the cytoskeleton (microtubules, actin filaments and intermediate filaments) (Donato 1999). Finally, several members of the S100 family interact with specific annexins, which in turn are targets of several kinases and are therefore thought to play an important role in intracellular signal transduction (Schafer & Heizmann 1996,Donato 1999).

Of particular interest is the upregulation of the S100P gene in the ErbB2 overexpressing cells. This gene has been previously reported to be upregulated in C3.6 cells by our lab (White *et al.* 2004), and its overexpression has been implicated in cellular immortalization and transformation of mammary epithelial cells (Guerreiro, I *et al.* 2000) and specifically related to ErbB2 status (Perou *et al.* 2000,Sorlie *et al.* 2001). S100A4 has also been reported to be up-regulated in cancers (Mazzucchelli 2002) and indeed in the C3.6 cells in the study by Mackay *et al* (Mackay *et al.* 2003). Despite this, there was little difference in S100A4 expression measured here (Figure 3.30) and so it is not possible to determine whether indeed ErbB2 regulates its expression.

The remaining S100 protein genes (S100A11, S100A2 and S100A6) were present in the "EGF only" gene list, with S100A2 showing a high level of induction in response to EGF only in HB4a cells. There is indeed evidence that EGF can

induce the expression of S100A2 (Stoll *et al.* 1998), reinforcing the results presented here. Both S100A2 and S100A6 have been shown to play a role in cell cycle regulation - S100A2 is a candidate tumour suppressor gene (Wicki *et al.* 1997) and inhibition of S100A6 was shown to result in reduced cellular proliferation (Breen & Tang 2003) - whereas S100A11 has been shown to have a potential role in endocytosis when complexed with annexin 1 (Seemann *et al.* 1997). While the functional significance of these changes in S100 gene expression is unclear, it is possible that they are responsible for eliciting some ErbB signalling-induced responses and/or play a role ErbB2-depedent tumourigenesis.

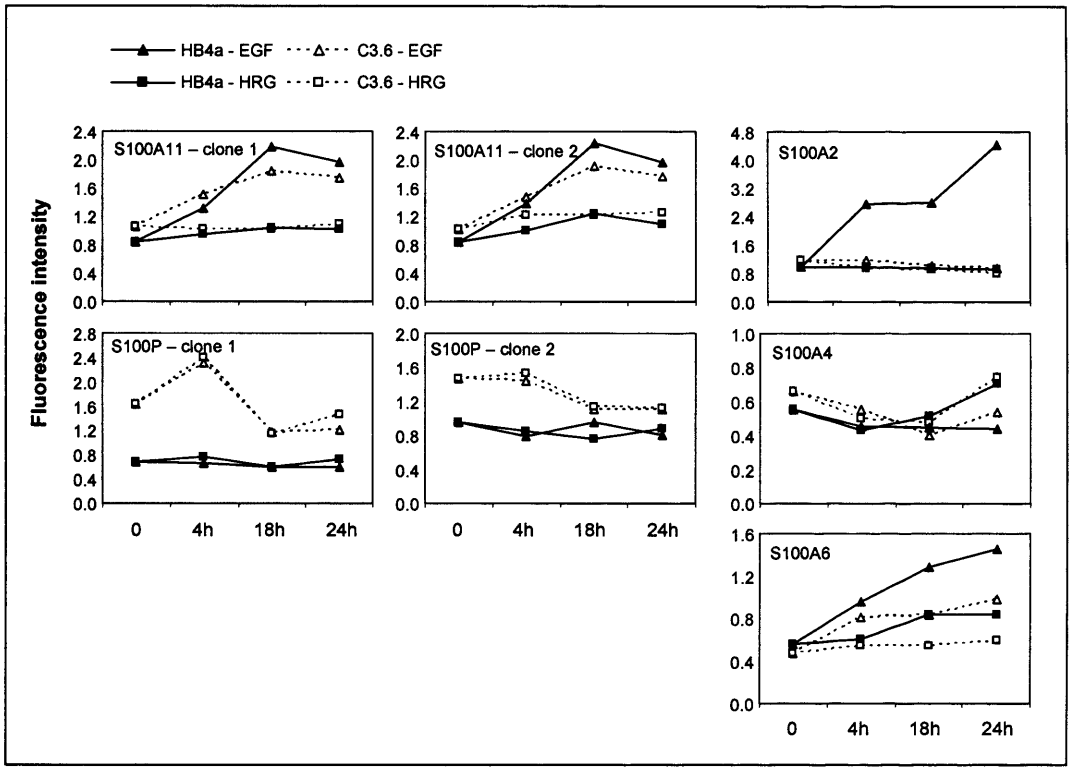

Figure 3.30: Expression profile of S100 proteins. Fluorescence intensity of genes encoding S100 proteins identified by SAM show a distinct expression pattern for each family member, reflecting the diverse role these proteins play in the cell.

3.8 Chapter Conclusions & Discussion

The growth of breast cancer cells can be regulated by growth factors that control proliferation, migration and apoptosis through the activation of various cell surface receptors. The ErbB receptor family plays a pivotal role in the development of breast cancer as its members can activate a multitude of cell signalling pathways that regulate many aspects of cellular function. In particular, ErbB2 is known to confer a more aggressive breast cancer phenotype with increased metastatic rates and markedly worse patient prognosis. Tumorigenesis is increasingly recognized as a process that involves the coordinated action of a group of genes, rather than of a single gene. Molecular events leading to malignant transformation of the breast involve changes in the expression of many oncogenic and tumour suppressor genes, leading to an unbalanced growth that results from high rates of proliferation as well as a tendency to better survive harmful stimuli which would otherwise lead to apoptosis. Thus, it is of great interest to identify key genes whose expression is altered, as a result of abnormal signalling, in ErbB2 overexpressing breast cells. Such abnormally expressed genes could lead to the development of breast cancer and/or the increased metastatic potential observed in such cancers, and therefore may represent important therapeutic markers for this disease. This Chapter describes the use of microarray technology, a powerful global gene expression profiling technique, in an attempt to try and identify such genes. In addition, analysis of gene expression changes associated with signalling pathways activated by EGF and HRG, two major growth factors that signal through ErbB receptors, are also described.

Using SAM as a statistical tool to identify significant changes in gene expression between parental and ErbB2-overexpressing cells, as well as between untreated cells and cells stimulated over a time course with EGF and HRG, 775 genes were identified. These genes were separated in a Venn diagram according to the statistical significance (as assigned by SAM) of changes in their expression according to whether it was affected by ErbB2 overexpression, EGF, HRG or a combination of these. A table with each of these gene lists is shown in Appendix 1. The transcriptional changes observed here are interesting in the context of breast cancer development and specific growth factor signalling.

The identified genes were grouped into major functional categories, including genes involved in cell cycle regulation, cell signalling, metabolism, protein transport, protein modification, cytoskeleton, adhesion and IFN signalling. Of particular

interest was the downregulation of multiple IFN-stimulated genes in the ErbB2-overexpressing cells. These genes have been implicated in the negative regulation of the cell cycle, and as such their downregulation may represent a mechanism by which ErbB2 overexpression due to gene amplification provides a proliferative advantage. Further research into the role of IFNs in breast cancer and cross-talk with ErbB2 is described in Chapter 5.

A number of the genes discussed above have been previously described as putative markers of cancers in a number of microarray studies. Two major publications need to be discussed in the context of the present study because they also analysed microarray expression data using the HB4a and C3.6 cellular model of ErbB2 overexpression. Mackay *et al* (Mackay *et al.* 2003) investigated transcriptional changes in randomly growing HB4a and C3.6 cells as well as an additional clone, C5.2, which expresses even higher levels of ErbB2. Using the reference microarray design, they identified 132 genes that showed statistically significant changes in expression in both C3.6 and C5.2 when compared to HB4a, including S100P, S100A4, CDH3, fibronectin, IGFBP3, EMP1 and TAGLN. All these genes were identified here and also showed similar directionality in terms of under- or overexpression. The study by White *et al* (White *et al.* 2004) looked at transcriptional changes in serum-starved HB4a and C3.6 cells and also analysed the effect of a time-course HRG stimulation on these cell lines. A large degree of overlap is observed between the two studies, and indeed with the results presented here, when the differences in expression between HB4a and C3.6 cells are taken into consideration. A number of other genes found to be significant in the latter study, but not on the former, include CPNE3 and various IFN-related genes, also identified here. The comparison between the HRG data of White *et al* and the data presented here is complicated by the different experimental designs used in these studies, as discussed in Section 3.3. Despite the high level of similarity between the three studies, a few discrepancies are also observed. Such variation can occur as a result of experimental design, hybridization artefacts, normalization and data analysis techniques and even differences in cell culture conditions. This further emphasizes the need to carefully interpret and validate microarray data, as variations can be found even when the same cell lines are studied.

To the best of our knowledge, this is the first study to simultaneously investigate global changes in gene expression due to ErbB2 overexpression as well

as growth factor signalling using two major ligands that are known to activate important pathways downstream of ErbB receptors in the same model cell system. Fully understanding and characterizing all the interactions and outcomes of this system is still a massive undertaking that requires a large effort and more experiments in order to validate the findings and allow definitive conclusions to be made about the role of these genes in tumourigenesis. However, while the findings presented in this Chapter represent the earliest stages in the search for breast cancer therapies, they provide a number of testable hypotheses with potentially important implications in the breast cancer field. It is indeed likely that not all genes identified here are directly involved in breast cancer development and progression, and may in fact show altered expression as a secondary consequence of upstream changes in protein function due to altered expression of other genes downstream of ErbB receptor signalling. In addition, a number of genes not represented on the array are likely to also contribute to the cellular processes that lead to cancer development. Many genes identified here are of unknown function, which further complicates data interpretation and the characterization of such genes and the processes they are involved in is beyond the scope of this project. Thus, further work on the results presented here are necessary in order to put such genes into a biologically significant context.

The advances made possible by microarray analysis have resulted in an extraordinary amount of information, often displaying a high degree of discrepancy between datasets, which can be overwhelming and difficult to summarize. There are many challenges in the microarray research field, and one of them is to integrate the large amount of data generated from different laboratories around the world into a single meaningful set of genes with real implication in breast cancer biology and therapeutics. In addition, tumour samples often contain a mixture of epithelial and stromal cells, blood vessels, adipose and connective tissue. Whilst cell lines isolated from tissues may represent a purer population, potentially important information regarding inter-cellular cross talk and tumour microenvironment may be compromised. Finally, microarray technology provides no information at the protein translation level. The identification of novel targets for therapeutic intervention using microarray data is therefore not a straightforward process. Nonetheless, the results presented here provide a number of potential targets for further testing that may prove to be important mediators of ErbB2-dependent breast cancer.

Chapter 4: MICROARRAY TARGET VALIDATION

4.1 Chapter Introduction

Global gene expression analysis using microarray technology is a powerful way to study the changes in gene expression within a cell. Differential data analysis using multiple samples leads to the identification of sets of genes that may play important roles in the biological system being studied. However, microarray analysis still generates potential artefacts and false positive calls with regard to the differentially expressed genes. Array results can be influenced by each step of the assay, from array manufacturing and sample preparation (extraction, labelling, and hybridization) to image analysis and data mining. As a result, the data generated from microarrays, although substantial, may benefit from validation with a more reproducible assay, ideally using a different analytical platform.

Chapter 3 described the microarray data analysis of a breast cancer model cell system that led to the identification of genes whose expression is altered in response to ErbB2 overexpression and/or specific growth factor stimulation. In this Chapter, a set of genes selected from this analysis were chosen to be further tested and validated using quantitative real time RT-PCR. In addition, the correlation between mRNA expression data and protein expression data obtained from parallel proteomics experiments and various western blotting experiments carried out in our laboratory was also investigated.

4.2 Introduction to Real-Time PCR Technology

As discussed above, it is essential that microarray results are validated independently. Commonly used methods for validating microarray data include conventional reverse transcription polymerase chain reaction (RT-PCR), northern blot, RNase protection assay and *in situ* hybridization or immunohistochemistry using tissue microarrays (Chuaqui *et al.* 2002). These assays can be time consuming and require large amounts of RNA. RT-PCR can be done using smaller amounts of RNA, but quantification is difficult as it relies on the endpoint analysis of the PCR product. Quantitative real time RT-PCR (hereafter referred to as qRT-PCR) measures product amplification during the initial log-linear phase of the reaction and is currently the most sensitive and accurate approach to gene expression quantification

(Wang & Brown 1999). Indeed, Rajeevan *et al* used a real time RT-PCR method to evaluate array data and they showed that the majority of array results were qualitatively accurate, indicating that qRT-PCR is a suitable technique to validate microarray results (Rajeevan *et al.* 2001).

The qRT-PCR technology capitalizes on the fact that there is a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given PCR cycle number. Starting material for qRT-PCR experiments consists of total RNA samples, which are reverse transcribed using random primers to generate cDNA samples which are then amplified by PCR using gene-specific primers. A number of detection chemistries have been developed that allow the real time quantification of PCR products throughout the amplification cycles. These include molecular beacons, scorpions, hybridization probes and double stranded DNA-binding dyes (such as SYBR Green). Here, the *Taq* polymerase hybridization probe system is described in more detail as it was used in the present study. This system relies on the 5' to 3' exonuclease activity of *Taq* DNA polymerase and on the construction of dual-labelled gene-specific oligonucleotide probes which only emit a fluorescence signal when cleaved by the exonuclease. This sequence-specific probe, also known as a TaqMan probe, is dually labelled with a reporter dye (FAM, or 6-carboxyfluorescein, used here) at one end of the probe and a non-fluorescent quencher at the 3' end. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence. During PCR, the TaqMan probe anneals to a complimentary sequence between the forward and reverse primer sets. As the target sequence is amplified, *Taq* polymerase enzyme cleaves the probe, which then separates the reporter dye from the quencher and allows the reporter dye to fluoresce. This results in an increase in reporter dye fluorescence with each amplification cycle. The increase in fluorescence emission can be read by a sequence detector in real time during the course of the reaction, and is a direct consequence of target amplification during PCR ((Giulietti *et al.* 2001) and Applied Biosystems white paper[††]). The principle of qRT-PCR is illustrated in Figure 4.1. The advantage of using fluorogenic probes is that non-specific amplification due to mis-priming or primer-primer artefacts does not generate a

[††] http://docs.appliedbiosystems.com/pebiodocs/00106737.pdf

fluorescence signal, although different probes must be synthesized to detect different genes, making it a more expensive option than other systems.

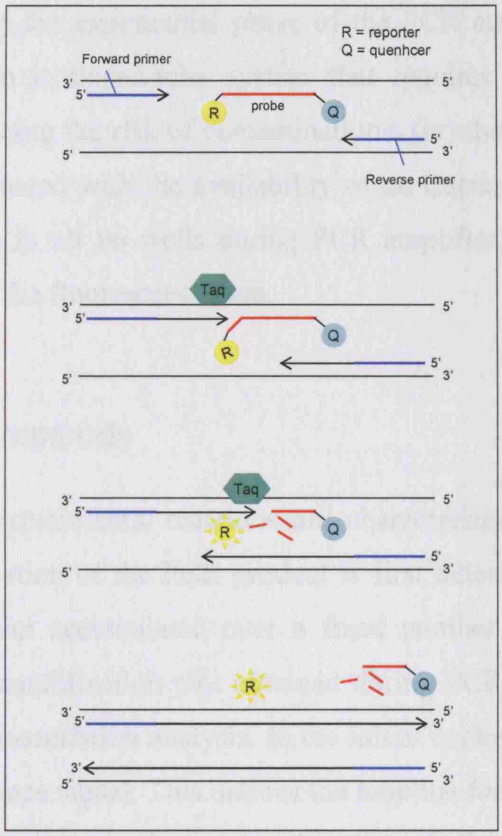

Figure 4.1: TaqMan qRT-PCR system (adapted from Applied Biosystems white paper‡‡). This figure shows the mechanism of probe cleavage resulting in fluorescence emission during one PCR cycle. After primer denaturation, primers and probe anneal to the target. Fluorescence does not occur because of the proximity of the reporter dye to the quencher. During the extension phase of the PCR cycle, the probe is cleaved by the 5' to 3' exonuclease activity of the Taq polymerase enzyme. The reporter and quencher are separated and fluorescence emitted. This process is repeated over many cycles and the fluorescence signal is increased with each PCR cycle.

‡‡ http://docs.appliedbiosystems.com/pebiodocs/00106737.pdf

The qRT-PCR method offers several advantages over other quantitative gene expression methods (Bieche *et al.* 1998): (i) the probe-based assay provides a real-time method for detecting only specific amplification products, since hybridization of both the primer and the probe is necessary to generate a fluorescence signal; (ii) the value used for quantitation (Ct value, discussed below) is measured when amplification is still in the exponential phase of the PCR amplification cycle; (iii) this method is run on a closed-tube system that requires no post-PCR sample handling, thereby reducing the risk of contaminations; (iv) the assay is rapid and the system is highly automated with the availability of an instrument that continuously measures fluorescence in all 96 wells during PCR amplification and software that can be used to analyse the fluorescence data.

4.2.1 Quantitation methods

In qRT-PCR experiments, reactions are characterized by the point during cycling when amplification of the PCR product is first detected, rather than by the amount of PCR product accumulated over a fixed number of cycles. Figure 4.2 shows a hypothetical amplification plot obtained during PCR amplification and the terminology used for quantitation analysis. In the initial cycles of PCR, there is little change in the fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product, and a fixed fluorescence threshold can be set above the baseline. Threshold cycle (Ct) values are then calculated by determining the point at which the fluorescence exceeds this chosen threshold limit. The Ct value is therefore defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline (Ginzinger 2002). The higher the starting copy number of the target, the earlier a significant increase in fluorescence is observed. Thus, Ct values decrease linearly with increasing input target quantity. The Ct values of different samples are then used to calculate the relative abundance of template for each sample (discussed in the next Section).

When quantitating gene expression in qRT-PCR experiments, it is very important to use an internal control gene to normalize the PCR for variations, regardless of the quantitation method used. Such PCR variations include differences

in the input amount of total RNA or in the efficiency of the reverse transcription reaction. Commonly used genes for these purpose are standard housekeeping genes, such as glyveraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin or 18S rRNA. However, a number of studies have shown that GAPDH and β-actin are not suitable internal controls for qRT-PCR normalization as their expression was shown to be influenced by experimental conditions, with 18S rRNA being the preferable housekeeping gene for qRT-PCR experiments (Schmittgen & Zakrajsek 2000,Goidin *et al.* 2001,Selvey *et al.* 2001). Thus, internal control genes must be carefully selected and validated for each experiment to determine that gene expression is not affected by the experimental treatment.

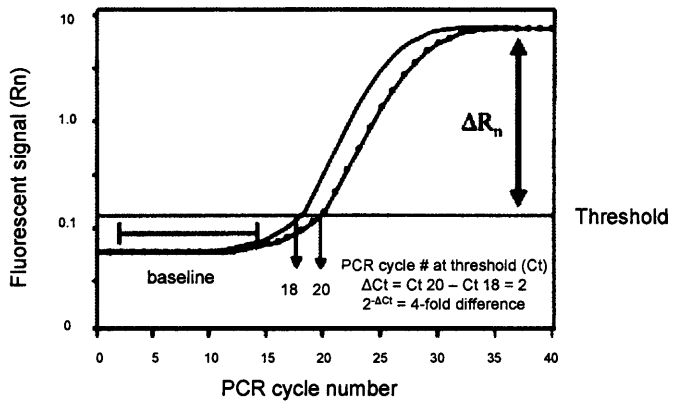

Figure 4.2: Representation of an amplification plot obtained in qRT-PCR experiments (from: (Ginzinger 2002)). The amplification plot of fluorescence signal vs. PCR cycle number is shown. The baseline indicated the PCR cycles in which fluorescence signal is below the detection limits of the instrument. This is used to set a fixed threshold, above which a signal detected is used to define the threshold cycle (Ct) value for a sample. Ct values are then used to determine the relative abundance of template for each sample based on the differences in Ct values between samples (ΔCt). Due to the exponential nature of PCR the ΔCt is converted to a linear form by calculating $2^{-\Delta Ct}$ to get relative fold-changes in expression.

There are two types of quantitation methods for qRT-PCR: relative quantitation and standard curve quantitation. The latter has also been named "absolute quantitation", but this is somewhat misleading as relative quantitation is also possible from standard curve methods when the exact DNA concentration of the standards used is not known (see Applied Biosystems user bulletin #2[§§]). Relative quantitation is the choice of quantitative analysis for most qRT-PCR studies, as in most studies absolute quantitation of copy number is not essential to obtain reliable results. The first method for relative quantitation of gene expression is the ΔCt method. In this method, arithmetic formulas are used to calculate the relative expression levels of the target gene within a sample to that of a control sample (or calibrator). To normalize the amount of target to an endogenous housekeeping gene, the Ct value of the target gene is subtracted from the Ct value of the housekeeping gene, giving ΔCt (target). The calibrator sample is also normalized to the housekeeping gene and the fold change of target sample relative to the calibrator sample is then given by $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt (target) – ΔCt (calibrator) (Livak & Schmittgen 2001). This value therefore represents the normalized expression of the target gene in an unknown sample relative to the normalized expression of the control (or calibrator) sample. This calculation is illustrated in Figure 4.2, but note that target normalization to a housekeeping gene is not shown. Although this method can be faster to perform (due to the fact that there is no standard curve and therefore more genes can be analysed in the spare wells), it requires that the PCR efficiencies of the target gene and the internal control gene are approximately equal. In addition, the assumption must be made that the chosen control gene does not vary in copy number or expression levels among the samples being studied.

In the second relative quantitation method, or the relative standard curve method, a standard curve for both the gene of interest and of a housekeeping gene is constructed by preparing serial dilutions of starting amounts of a known sample. The known sample can be purified plasmid DNA, *in vitro* transcribed RNA, *in vitro* synthesized RNA, or any cDNA sample expressing the target gene (Giulietti *et al.* 2001). The concentration of these DNA and RNA samples can be measured in a spectrophotometer at 260 nm and converted to the number of copies using the molecular weight of DNA or RNA. This enables the absolute quantitation of

[§§] http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf

unknown samples. If the concentration of the sample used in the standard curve preparation is not determined, the quantitation of the unknown samples will be made relative to a control, or a calibrator sample, as long as the fold dilutions are known. In this case, the sample is used to create a dilution series with arbitrary units. To calculate the relative abundance of a gene of interest the following is performed: (i) for each experimental sample, the amount of target and housekeeping gene is determined from the appropriate standard curve; (ii) the target amount is divided by the housekeeping amount to obtain a normalized target value; (iii) one of the experimental samples is selected as the calibrator; (iv) each of the normalized target values is divided by the calibrator normalized value to generate the relative levels of expression. Thus, all that is required of the standards is that their relative dilutions be known, and the normalized amount of target is a unitless number where all quantities are expressed as an n-fold difference relative to the calibrator (Bustin 2000,Giulietti *et al.* 2001).

4.3 Validation of Microarray Data by qRT-PCR

For this validation study, HB4a and C3.6 cells were freshly seeded and stimulated under the same conditions as in the microarray experiments. Cell lines were left serum-starved or stimulated with EGF or HRG for 4hr, 18hr or 24hr. Total RNA was isolated from cells using the same protocol as previously described, quantitated, quality controlled and reverse transcribed to generate cDNA samples for the qRT-PCR experiments. The same cDNA samples were used for all qRT-PCR experiments described in this Chapter. Gene expression was quantitated using the relative standard curve method, and the fold-change for each gene was calculated relative to the untreated HB4a cells (calibrator). While GAPDH was the initial choice of internal control, it was found to be responsive to the experimental conditions being studied here (Figure 4.3-a and see previous Section). All samples were therefore normalized to the 18S rRNA housekeeping gene, which showed minimal variation between the samples being analysed (Figure 4.3-a). The standard curve for both the target gene and the housekeeping gene was constructed using serial dilutions of the same sample that was used as the calibrator (HB4a control). An example of standard curves obtained is shown in Figure 4.3-b, together with the graph derived from it. The R^2 value should be as close to 1 as possible, and the values obtained

from the curve in the equation in the form of $y = mx + b$ (where m = the slope of the standard curve line and b = y-intercept of standard curve line) are used to calculate the input amount for unknown samples (this data analysis protocol can be found on Applied Biosystems user bulletin number 2[***]). Standard curves were constructed based on triplicate Ct values from different wells, whereas unknowns were measured in duplicate. Ideally, at least triplicates should be run to obtain more statistically significant results. However, when the unknown samples were run in duplicate, extremely low levels of variation were observed in the majority of experiments. This is exemplified in Figure 4.3-c, which shows the amplification plot obtained for a gene known to be differentially expressed between HB4a and C3.6 cells. The differences are clear from the graph, whilst the duplicate amplification plots for each cell line are identical with extremely small variations in the Ct values obtained. In a very few cases, the variation between duplicates was slightly higher. For this reason, error bars have been plotted to allow the visualization of variation between replicates.

[***] http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf

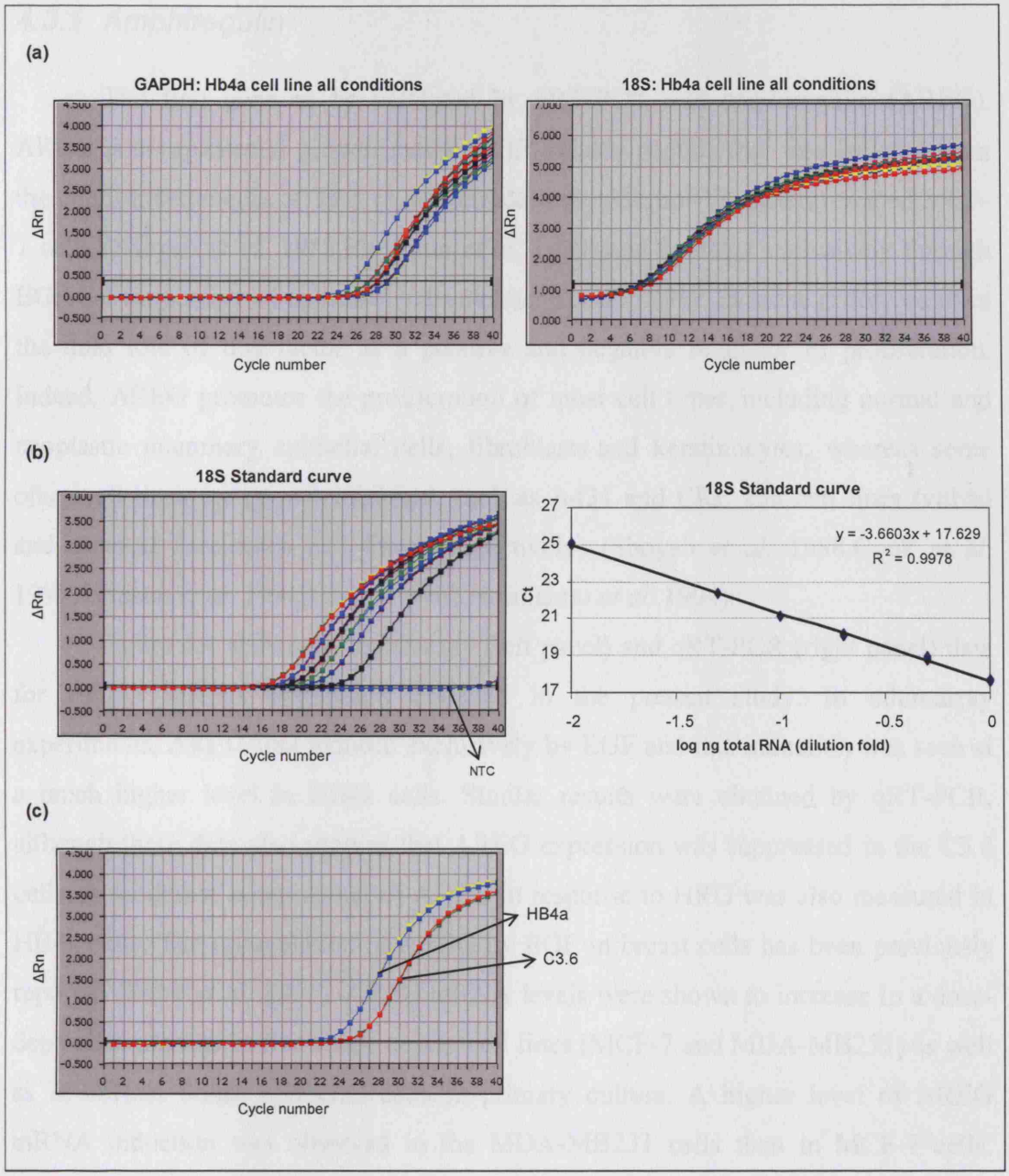

Figure 4.3: Examples of qRT-PCR amplification plots obtained in the present study. (a) HB4a cell line control and stimulated samples were amplified using specific primers for the GAPDH and 18S housekeeping genes. GAPDH showed considerable variation in expression levels with growth factor treatment (left panel), whereas 18S showed very little variation in expression among samples. These results were also observed in C3.6 cells (data not shown). **(b)** Serial dilutions of the calibrator samples were used to construct the standard curve, shown here for the 18S gene. The graph and equation derived from the results obtained (right panel) is used to calculate the input amount for each sample. Non-template controls (NTC) are run to test for non-specific fluorescence emission. **(c)** Amplification plot of a target gene in duplicate (in this case p48) showing differences in expression level between the HB4a and C3.6 cell lines. Note the accuracy of the duplicate samples run in separate wells.

4.3.1 Amphiregulin

The first gene to be validated by qRT-PCR was amphiregulin (AREG). AREG is an epidermal growth factor (EGF)-related peptide that was isolated from the conditioned media of TPA (12-*O*-tetradecanoylphorbol-13-acetate)-treated MCF-7 cells (Shoyab *et al.* 1988,Plowman *et al.* 1990) and operates exclusively through EGFR at the surface of epithelial cells (Johnson *et al.* 1993). Its name is derived from the dual role of this factor as a positive and negative regulator of proliferation. Indeed, AREG promotes the proliferation of most cell types, including normal and neoplastic mammary epithelial cells, fibroblasts and keratinocytes, whereas some other cell lines are growth inhibited, such as A431 and CRL 155 cell lines (vulval and cervical carcinoma cell lines, respectively) (Shoyab *et al.* 1988,Cook *et al.* 1991,Johnson *et al.* 1991,Li *et al.* 1992,Normanno *et al.* 1994).

Figure 4.4 shows the microarray (left panel) and qRT-PCR (right panel) data for AREG mRNA expression obtained in the present study. In microarray experiments, AREG was induced exclusively by EGF and this induction was seen at a much higher level in HB4a cells. Similar results were obtained by qRT-PCR, although these data also showed that AREG expression was suppressed in the C3.6 cells. A moderate upregulation of AREG in response to HRG was also measured in HB4a cells. The upregulation of AREG by EGF in breast cells has been previously reported (Silvy *et al.* 2001). AREG mRNA levels were shown to increase in a dose-dependent manner in two breast cancer cell lines (MCF-7 and MDA-MB231) as well as in normal breast epithelial cells in primary culture. A higher level of AREG mRNA induction was observed in the MDA-MB231 cells than in MCF-7 cells. Interestingly, MDA-MB231 cells express higher levels of EGFR then MCF-7 cells, which express more ErbB3 and very low levels of other ErbB family members ((deFazio *et al.* 2000) and data from our laboratory). This data, together with the elevated induction of AREG in HB4a cells (which express high EGFR levels compared to C3.6 cells), indicates that upregulation of AREG by EGF is mediated primarily by EGFR and may act as a positive feedback (autocrine) loop.

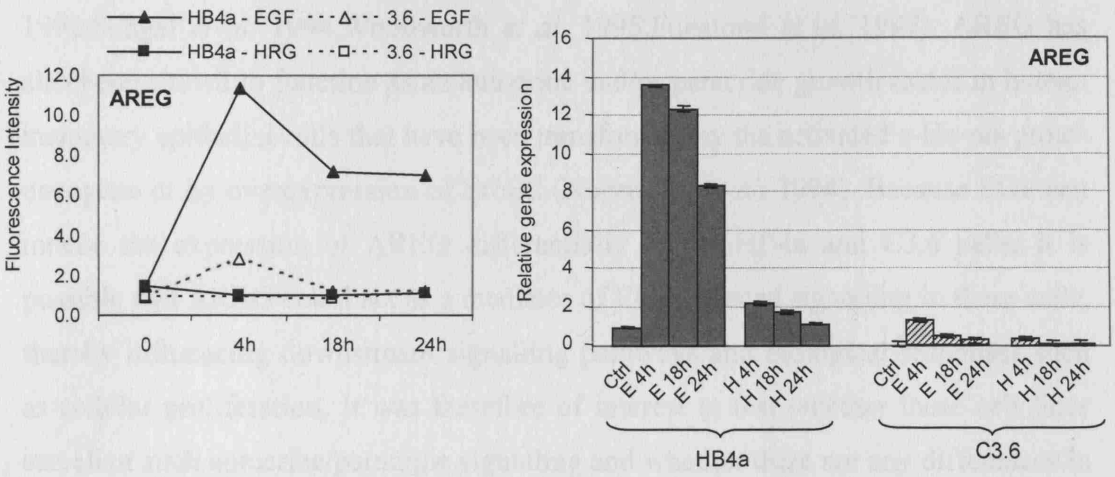

Figure 4.4: Comparison of gene expression levels of AREG between microarray and qRT-PCR experiments. The left panel shows the expression levels of AREG obtained by microarray. Values represent fluorescence intensity from normalized microarray data. The right panel shows the results obtained by qRT-PCR experiments. Values represent the relative expression levels, or fold change, for each sample relative to that of untreated HB4a cells (calibrator). E: EGF, H: HRG.

The relevance of these findings in understanding the progression of ErbB2-positive breast cancers is not yet clear, and there is controversy in the literature regarding the role of AREG expression in breast cancer. In one study, no significant correlation between AREG and ErbB2 overexpression in primary breast cancers was found (LeJeune *et al.* 1993). However, another report showed that there was increased AREG mRNA expression in normal human mammary epithelial cells compared to tumour-derived cells in culture (Li *et al.* 1992), whereas immunohistochemistry studies of primary infiltrating ductal and lobular carcinomas showed overexpression of AREG (Qi *et al.* 1994). More recently, a study revealed that AREG showed no prognostic relevance as no correlation was found between AREG expression and disease recurrence or overall survival in 193 ductal and invasive lobular breast carcinoma patients (Desruisseau *et al.* 2004). Although AREG is known to induce the phosphorylation of ErbB2 in cells co-expressing EGFR (Johnson *et al.* 1993,Riese *et al.* 1996), the role of ErbB2 in regulating the expression of AREG has not been established.

AREG has been shown *in vitro* to function in an autocrine manner to drive the proliferation of malignant cells of colon, cervix, prostate, and pancreas (Johnson *et al.*

1992,Sehgal *et al.* 1994,Woodworth *et al.* 1995,Funatomi *et al.* 1997). AREG has also been shown to function as an autocrine and/or paracrine growth factor in human mammary epithelial cells that have been transformed by the activated c-Ha-ras proto-oncogene or by overexpression of ErbB2 (Normanno *et al.* 1994). Because EGF can induce the expression of AREG differentially in the HB4a and C3.6 cells, it is possible that AREG could act as a mediator of EGF-induced signalling in these cells, thereby influencing downstream signalling pathways and biological responses such as cellular proliferation. It was therefore of interest to test whether these cell lines can elicit such autocrine/paracrine signalling and whether there are any differences in the activation of downstream signalling pathways. Figure 4.5 shows "media-swap" experiments where serum-starved HB4a and C3.6 cells were treated with conditioned media (CM) from serum-starved, EGF-, serum- or HRG treated cells for 15 minutes. CM was made by stimulating cells with serum or growth factors for 4 hours followed by extensive washing of plates with PBS and addition of fresh, serum-free media for a further 4 hours to allow secretion of molecules into the media. Activation of MAPK signalling was then monitored by immunoblotting with a phospho-specific antibody for ERK1/2. Addition of CM from both cell lines could trigger the activation of MAPK signalling, which was higher in the treated HB4a cells. In addition, EGF-generated CM was a more potent activator of MAPK signalling than HRG- and serum-generated CM (Figure 4.5-a). Importantly, pre-treatment of cells with the ErbB kinase inhibitor AG1478 inhibited this response (Figure 4.5-b), confirming that growth factors such as EGF and HRG can induce the secretion of autocrine ligands such as AREG which activate signalling through ErbB receptors. The difference in MAPK activation between the two cell lines and between growth factors is likely to be due to the differential level of ErbB receptor expression, with autocrine/paracrine signalling occurring primarily in an EGFR-dependent fashion.

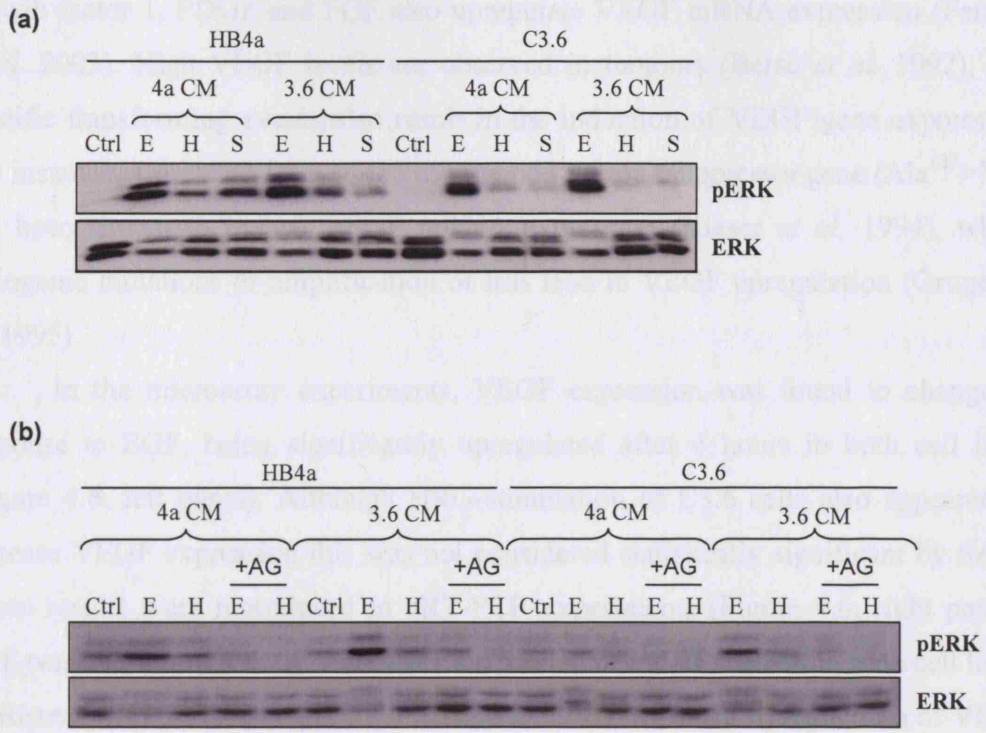

Figure 4.5: Analysis of autocrine/paracrine activation of MAPK pathway in HB4a and C3.6 cell lines. (a) Serum-starved HB4a and C3.6 cells were treated with EGF (E), HRG (H) or fetal calf serum (S) for 4 hours. Cells were washed extensively and the media was then replaced with serum-free media for 4 hours to allow secretion of factors into the media. This conditioned media (CM) was then added to freshly plated, serum-starved cells for 15 minutes. Activation of MAPK pathway was measure by immunoblotting using antibodies specific for the phosphorylated form or ERK1/2. Non-phospho ERK1/2 was used as a control. **(b)** CM was prepared as in (a) using EGF and HRG. Freshly plated, serum-starved HB4a and C3.6 cells were pre-treated with the ErbB inhibitor AG1478 (AG) for 1 hour prior addition of CM for 15 minutes.

4.3.2 VEGF

VEGF is a potent mitogen for vascular endothelial cells, the essential component of blood vessels, and plays a key role in the normal regulation of angiogenesis as well as pathological angiogenesis, such as tumour-associated neo-vascularization. The VEGF gene product is alternatively spliced, giving rise to four distinct VEGF proteins of 121, 165, 189, and 206 amino acids (Tischer *et al.* 1991). Hypoxia is considered to be the major inducer of VEGF gene expression (Dor *et al.* 2001), but several growth factors, including EGF, TGF-α, TGF-β, insulin-like

growth factor 1, PDGF and FGF also upregulate VEGF mRNA expression (Ferrara *et al.* 2003). High VEGF levels are observed in tumours (Berse *et al.* 1992), and specific transforming events also result in the induction of VEGF gene expression. For instance, a mutated form of the murine p53 tumour suppressor gene (Ala^{135}>Val) has been shown to induce VEGF mRNA expression (Kieser *et al.* 1994), whilst oncogenic mutations or amplification of Ras lead to VEGF upregulation (Grugel *et al.* 1995).

In the microarray experiments, VEGF expression was found to change in response to EGF, being significantly upregulated after 4 hours in both cell lines (Figure 4.6, left panel). Although HRG-stimulation of C3.6 cells also appeared to increase VEGF expression this was not considered statistically significant by SAM. These results were reproduced in qRT-PCR experiments (Figure 4.6, right panel); EGF was able to transiently induce the expression of VEGF mRNA in both cell lines, consistent with previous reports (Ferrara *et al.* 2003). A small upregulation of VEGF by HRG was seen in qRT-PCR experiments, consistent with reports showing that HRG induces VEGF secretion as a result of increased mRNA expression through the p38 signalling pathway (Yen *et al.* 2000,Xiong *et al.* 2001).

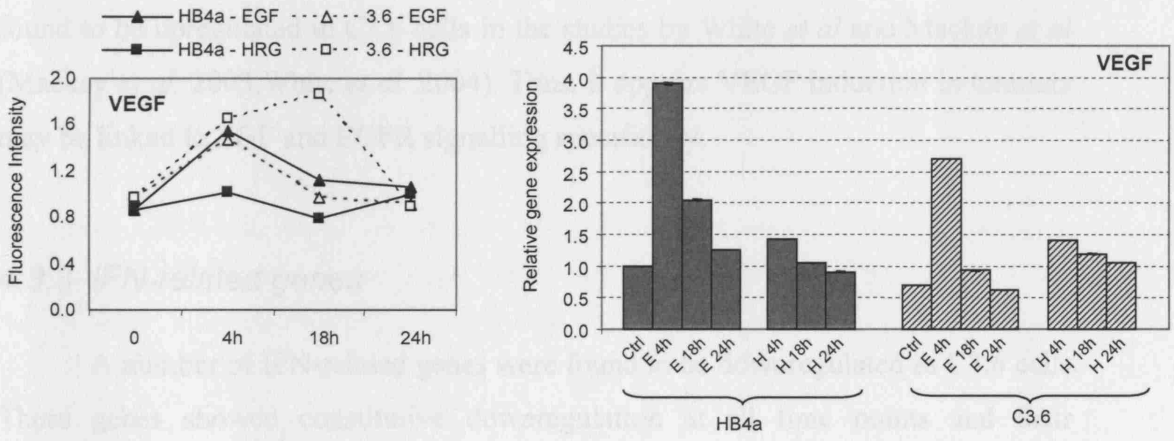

Figure 4.6: Transcriptional regulation of VEGF in microarray and qRT-PCR experiments. The left panel represents the microarray fluorescence intensity data for the VEGF gene. The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

No significant differences in VEGF levels were seen between control C3.6 and HB4a cells in either the microarray or qRT-PCR experiments, indicating that ErbB2 is not a direct regulator of VEGF gene expression. However, these findings contradict previous literature reports. HRG has been shown to induce VEGF mRNA upregulation when signalling through ErbB2 (Yen *et al.* 2000); ErbB2 overexpression was shown to lead to an increase in VEGF mRNA in a mouse mammary cell line (Loureiro *et al.* 2005); and treatment of ErbB2-overexpressing mammary tumour cell lines with an ErbB2-specific neutralizing antibody leads to a dose-dependent decrease in VEGF protein production (Petit *et al.* 1997). In addition, Herceptin, the commercially available monoclonal antibody currently used in breast cancer therapy, has anti-angiogenic properties against human mammary tumours implanted in mice (Izumi *et al.* 2002). These reports implicate ErbB2 in the regulation of VEGF expression and angiogenesis in breast cancers but are not consistent with the findings presented here. One possibility is that the C3.6 cell line does not represent the stage of breast cancer where angiogenesis takes place. Indeed, although C3.6 cells show ErbB2-dependent transformation *in vitro* (as shown by their enhanced anchorage-independent proliferation and ability to form colonies in soft agar) they are unable to produce tumours in nude mice (Harris *et al.* 1999), suggesting that further events or higher ErbB2 levels may be required for progression to full malignancy in human mammary epithelial cells. Moreover, VEGF was not found to be upregulated in C3.6 cells in the studies by White *et al* and Mackay *et al* (Mackay *et al.* 2003,White *et al.* 2004). Thus, it appears VEGF induction in tumours may be linked to EGF and EGFR signalling specifically.

4.3.3 IFN-related genes

A number of IFN-related genes were found to be downregulated in C3.6 cells. These genes showed constitutive downregulation at all time points and their expression was not significantly affected by growth factor stimulation. These findings suggest that these genes are basally suppressed by ErbB2 overexpression which could have important implications in the development of breast cancer. Indeed, IFNs are known to have negative effects on cellular proliferation (Sangfelt *et al.* 2000). One of the downregulated genes, p48 (ISGF3γ), is a member of the ISGF3

transcriptional complex, also composed of STAT1 and STAT2. This transcription factor complex is responsible for the induction of a large number of downstream type I IFN-stimulated genes (ISGs) that contain the ISRE sequence within their promoters (Bluyssen *et al.* 1996,Stark *et al.* 1998). Thus, it is possible that the downregulation of other ISGs is a consequence of the downregulation of p48 itself. As seen in Figure 4.7, p48 is indeed downregulated in C3.6 cells, with qRT-PCR experiments showing very similar patterns of gene expression to that observed in the microarray experiments. Interestingly, p48 seemed to be upregulated by growth factor treatment at the longer time points in the qRT-PCR results, particularly in EGF treated C3.6 cells. While growth factors are known to induce the phosphorylation and activation of STAT proteins to initiate signal transduction, their effect on the levels of p48 is less well studied. It is therefore difficult to assess the significance of such expression level changes found here. The effect of growth factor stimulation on p48 protein expression levels and on IFN signalling is further discussed in Chapter 5.

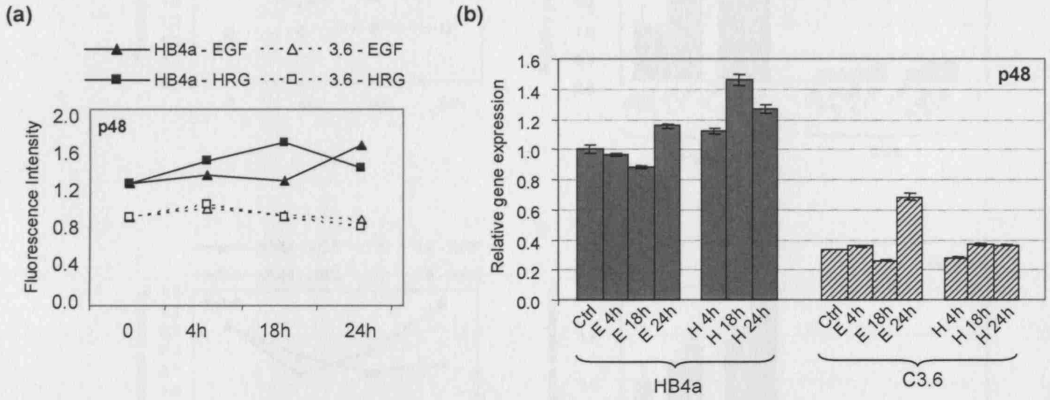

Figure 4.7: Downregulation of p48 in C3.6 cells. (a) Fluorescence intensities of p48 levels in the microarray data showing the downregulation of p48 in C3.6 cells. **(b)** Real time PCR data confirming the differences in expression levels of p48 between HB4a and C3.6 cells. HB4a untreated samples were used as the calibrator. E: EGF, H: HRG.

The levels of mRNA expression of other ISGs were also validated by qRT-PCR. Both OAS1 and G1P2 were downregulated in C3.6 cells and showed similar expression patterns in qRT-PCR experiments as in the microarray experiments (Figure 4.8). For these genes, in particular OAS1, there was a higher degree of

variability between duplicate samples making it difficult to determine the effect of growth factor stimulation of these genes. G1P2, also known as ISG15, was the most downregulated gene in the microarray dataset. This gene is known to be regulated transcriptionally, and type I IFNs (IFNα/β) are efficient at inducing its expression. Its promoter contains two ISRE sites, confirming that G1P2 can be induced by the ISGF3 complex (Ritchie & Zhang 2004). ISG15 plays an important role in protein modification in a process similar to that of ubiquitin, where it conjugates to intracellular proteins to modify their function, a process termed "ISGylation", a process induced mainly via the type I IFN signalling pathway (Loeb & Haas 1992).

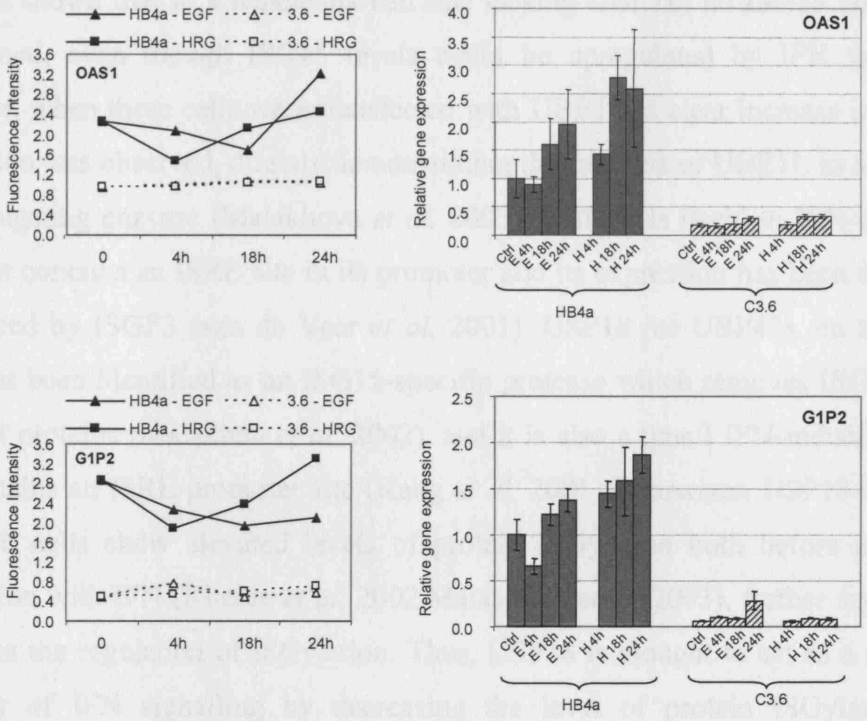

Figure 4.8: Gene expression levels of OAS1 and G1P2. Both genes were found to be significantly downregulated in C3.6 cells during microarray data analysis. Left panel shows the expression levels of OAS1 and G1P2 obtained from microarray experiments. The right panel shows similar expression patterns obtained in qRT-PCR experiments of these two genes. E: EGF, H: HRG

In order to test whether other ISG targets of p48 not identified in the microarray analysis were also downregulated in C3.6 cells, primers for SOD2, USP18 and UBE1L were tested. SOD2 and UBE1L were both represented on the array, but were not identified as significantly changing. This could be due to the lower sensitivity of microarray experiments or due to high variability among replicates. USP18 was not represented on the array. SOD2 was found to be downregulated in parallel proteomics experiments (Gharbi *et al.* 2002), and qRT-PCR results confirm that this downregulation occurs at the transcriptional level (Figure 4.9-a). SOD2 downregulation in C3.6 cells was not as marked as other ISGs, but it was induced by early EGF stimulation in HB4a cells. USP18 and UBE1L both work together with G1P2 contributing to the ISGylation of proteins. UBE1L is thought to act as an E1-like conjugating enzyme in the ISGylation process. Indeed, it has been shown that in a leukaemia cell line lacking UBE1L, no ISG15 conjugates are formed, even though ISG15 levels could be upregulated by IFN treatment. However, when these cells were transfected with UBE1L, a clear increase in protein ISGylation was observed, directly demonstrating the function of UBE1L as an ISG15 E1 conjugating enzyme (Malakhova *et al.* 2003). UBE1L is itself an IFN-inducible gene that contains an ISRE site in its promoter and its expression has been shown to be induced by ISGF3 (van de Veer *et al.* 2001). USP18 (or UBP43), on the other hand, has been identified as an ISG15-specific protease which removes ISG15 from its target proteins (Malakhov *et al.* 2002), and it is also a type I IFN-inducible gene that contains an ISRE promoter site (Kang *et al.* 2001). Moreover, USP18-deficient mice and cells show elevated levels of protein ISGylation both before and after stimulation with IFN (Ritchie *et al.* 2002,Malakhova *et al.* 2003), further supporting its role in the regulation of ISGylation. Thus, USP18 is thought to act as a negative regulator of IFN signalling by decreasing the level of protein ISGylation. As expected, both UBE1L and USP18 were highly downregulated in C3.6 cells (Figure 4.9-b). Interestingly, EGF induced a modest downregulation of UBE1L while causing upregulation of USP18 mRNA. This trend was seen in both cell lines. At the time of writing, there were no reports on the relationship between EGF and any of the genes involved in protein ISGylation. Further studies in the ISGylation process and how it is affected by ErbB signalling may provide additional information of the regulation of proliferation by ISGylation.

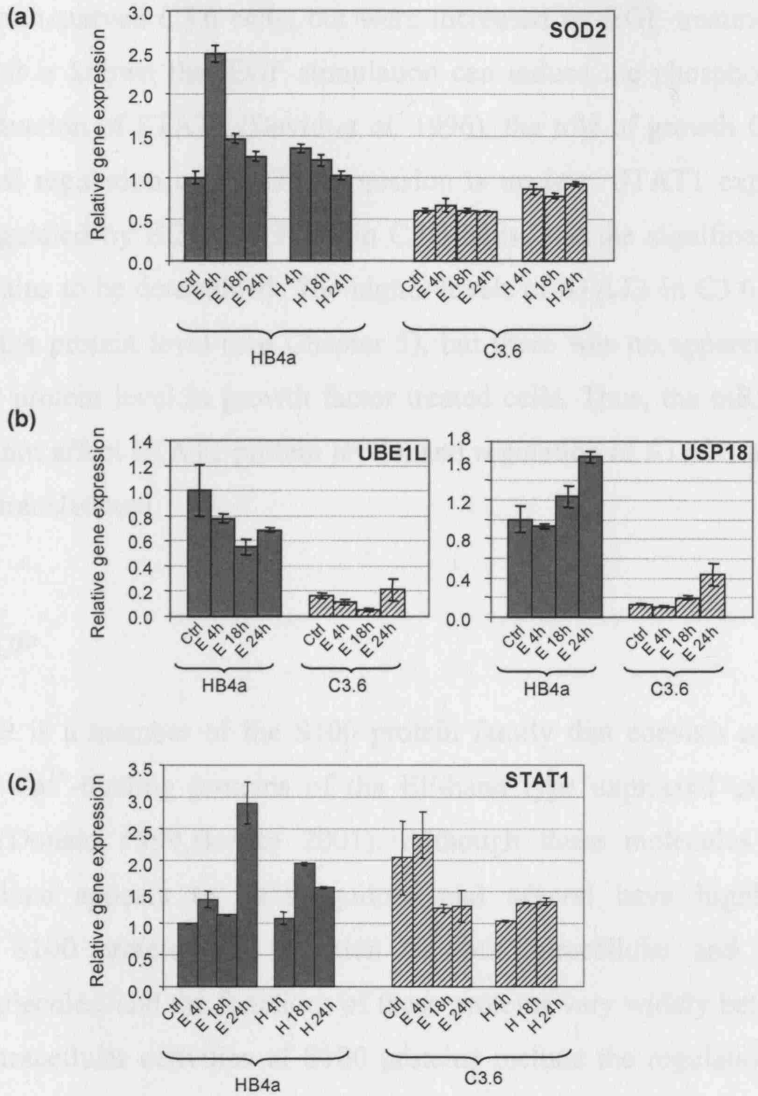

Figure 4.9: Gene expression levels of ISGs and STAT1 as determined by qRT-PCR. (a) Gene expression data for SOD2 showing downregulation in C3.6 cells. Compared to other ISGs, the fold-change in SOD2 expression between HB4a and C3.6 cells was small. **(b)** Confirmation of the downregulation of UBE1L and USP18, two ISGs thought to participate in the "ISGylation" of proteins mediated by G1P2. (c) Gene expression data for STAT1, showing its upregulation in C3.6 cells and variable expression in growth factor treated samples. E: EGF, H: HRG

STAT1 mRNA levels were also tested by qRT-PCR (Figure 4.9-c). In the microarray experiments, there was no difference in STAT1 levels between the two cell lines, but STAT1 was significantly upregulated by 18h HRG stimulation in both HB4a and C3.6 cells. In the qRT-PCR experiments, STAT1 mRNA levels were higher in serum-starved C3.6 cells, but were increased by EGF treatment in HB4a cells. While it is known that EGF stimulation can induce the phosphorylation and therefore activation of STAT1 (David *et al.* 1996), the role of growth factors in the transcriptional regulation of STAT1 expression is unclear. STAT1 expression was also downregulated by EGF and HRG in C3.6 cells, and the significance of these findings remains to be determined. The higher levels of STAT1 in C3.6 cells can be observed at the protein level (see Chapter 5), but there was no apparent change in total STAT1 protein level in growth factor treated cells. Thus, the mRNA changes observed do not affect STAT1 protein levels, and regulation of STAT1 appears to be mainly post-translational.

4.3.4 S100P

S100P is a member of the S100 protein family that consists of at least 20 members of Ca^{2+}-binding proteins of the EF-hand type expressed exclusively in vertebrates (Donato 1999,Donato 2001). Although these molecules are widely expressed, none appears to be ubiquitous and several have highly restricted distribution. S100 proteins can function as both intracellular and extracellular signalling molecules, and the functions of these proteins vary widely between family members. Intracellular activities of S100 proteins include the regulation of protein phosphorylation, cell growth, enzyme activity, cytoskeletal function, intracellular Ca^{2+} homeostasis and protection from oxidative stress. Whilst several S100s are known to be released from cells and to act extracellularly, the mechanisms of secretion are obscure (Schafer & Heizmann 1996,Donato 1999,Donato 2001).

S100P was chosen for validation by qRT-PCR because several lines of evidence have implicated it in cancer development and progression. S100P was shown to be overexpressed in prostate cancer in an androgen-sensitive manner (Averboukh *et al.* 1996) and in pancreatic cancers by microarray analysis (Iacobuzio-Donahue *et al.* 2002,Logsdon *et al.* 2003), and its expression has been correlated

with decreased survival in lung cancer patients (Beer *et al.* 2002). S100P overexpression has also been associated with cellular immortalization of human breast epithelial cells (Guerreiro, I *et al.* 2000), and was previously found to be overexpressed in the ErbB2-overexpressing C3.6 cells (Mackay *et al.* 2003,White *et al.* 2004). Therefore it was of interest to validate the differential regulation of S100P using a different gene expression assay, thereby providing further evidence for the role of S100P in breast tumourigenesis. The S100P gene was represented twice on the microarrays and after SAM statistical analysis each clone was placed in a different gene list. The pattern of expression for both S100P clones in the array is shown in Figure 4.10, left panel. While both clones were significantly upregulated in C3.6 cells, one clone was present in the "changing in all conditions" list (i.e. differentially regulated in C3.6 cells compared to HB4a and also responsive to both growth factors). The overexpression of S100P in C3.6 cells was confirmed by qRT-PCR (Figure 4.10, right panel), indicating that ErbB2 overexpression can increase S100P expression. Growth factor-induced downregulation of S100P was also reproduced in the qRT-PCR experiment. Very little is known about the regulation of S100P expression. S100P mRNA has been shown to be regulated by androgens, the factors that regulate the growth and function of the normal prostate (Averboukh *et al.* 1996,Amler *et al.* 2000), and more recently by interleukin 6 (IL6) (Hammacher *et al.* 2005), but no reports have been published linking growth factors and S100P expression. Thus, these results provide evidence for a novel mechanism of regulation of S100P gene expression and further reinforce the hypothesis that S100P is a target of ErbB2 overexpression.

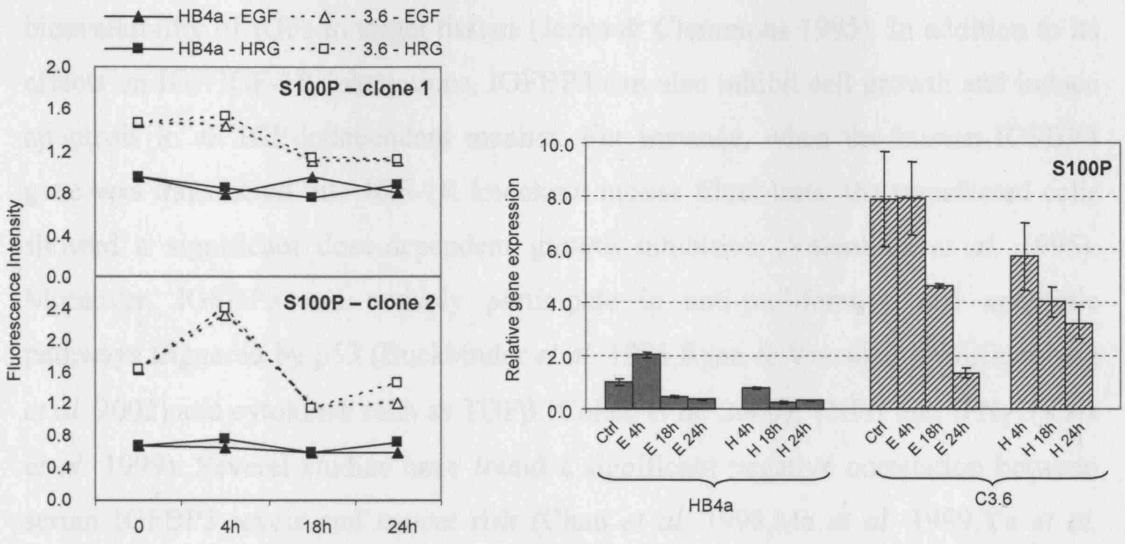

Figure 4.10: Transcriptional regulation of S100P in microarray and qRT-PCR experiments. The left panel represents the microarray data obtained for two clones of the S100P gene represented on the array. The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

4.3.5 IGFBP3

IGFBP3, or insulin-like growth factor binding protein 3, is a protein that associates with and modulates the activity of insulin-like growth factors I and II (IFG-I and IFG-II). IGFs are potent mitogenic and anti-apoptotic factors whose effects are mediated by type I IGF receptors (IGF-1R) at the cell surface. IGF-1R has been shown to stimulate mitogenesis in cultured cell lines (Esposito *et al.* 1997), to protect cells against apoptosis (O'Connor *et al.* 1997) and to be an essential component for cellular transformation by agents such as SV40 T-antigen (Sell *et al.* 1993). Over 90% of circulating IGFs are bound to IGFBP3. IGFs can also be carried by other IGFBPs, and less than 1% circulate in the free form. IGFBP3 further binds to another large protein called acid labile subunit (ALS). IGFBP3, IGF and ALS form a 150kDa ternary complex that is believed to be the major form of IGFs in circulation (Baxter 1994).

In most situations, IGFBP3 inhibits the action of IGFs by competing with the binding of IGF to IGF-1R to inhibit IGF-mediated survival signalling and therefore functions as a pro-apoptotic agent. In some cases, however, IGFBP3 can enhance IGF activity by protecting IGFs from degradation thereby increasing the

bioavailability of IGFs in target tissues (Jones & Clemmons 1995). In addition to its effects on IGF/IGF-1R interactions, IGFBP3 can also inhibit cell growth and induce apoptosis in an IGF-independent manner. For instance, when the human IGFBP3 gene was transfected into IGF-1R knockout mouse fibroblasts, the transfected cells showed a significant dose-dependent growth inhibition (Valentinis *et al.* 1995). Moreover, IGFBP3 can actively participate in anti-proliferative and apoptotic pathways triggered by p53 (Buckbinder *et al.* 1995,Ryan & Vousden 1998,Grimberg *et al.* 2002) and cytokines such as TGFβ (Cohen *et al.* 2000), TNFα and IFNγ (Katz *et al.* 1999). Several studies have found a significant negative correlation between serum IGFBP3 levels and cancer risk (Chan *et al.* 1998,Ma *et al.* 1999,Yu *et al.* 1999). Thus, IGFBP3 may serve a protective role against carcinogenesis.

Two clones representing the IGFBP3 gene were represented on the microarrays in the present study. These appeared in different gene lists following statistical data filtering. Both clones were highly downregulated in C3.6 cells and also following EGF stimulation in both cell lines, whilst one clone was more strongly downregulated by HRG (Figure 4.11, left panel). The gene expression pattern was similar in the qRT-PCR experiment (Figure 4.11, right panel); IGFBP3 was highly downregulated in C3.6 cells and its downregulation in response to growth factor, particularly EGF, was more potent in HB4a cells, although the timing of expression reduction was different for the two techniques.

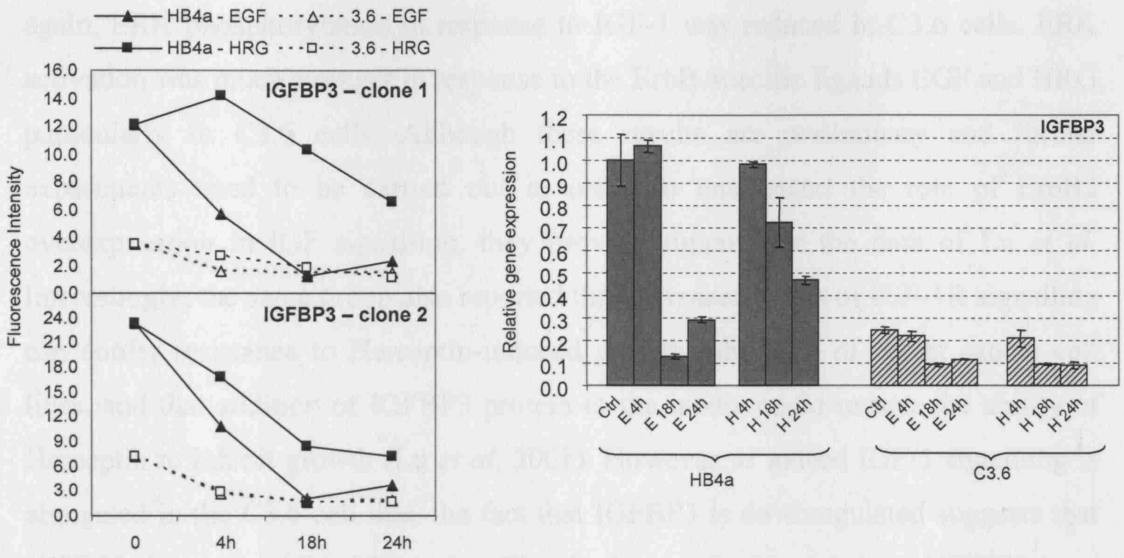

Figure 4.11: Transcriptional regulation of IGFBP3 in microarray and qRT-PCR experiments. The left panel represents the microarray data obtained for both clones of the IGFBP3 gene represented on the array. The expression of IGFBP3 measure by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

Although IGFBP3 is thought to have a protective role against tumourigenesis, there are no reports to date showing that altered IGFBP3 expression levels may have a role in ErbB2-dependent breast cancer progression. However, an interplay between the IGF-I and ErbB2 signalling pathways has been suggested. Lu *et al* (Lu *et al.* 2004) showed that IGF-1-induced activation of the MAPK pathway was inhibited by ErbB2 overexpression in breast cancer cells. To test this hypothesis in the HB4a and C3.6 cell lines, cells were serum-starved for 24 hours and stimulated with IGF-1 for various times and activation of MAPK was measured using antibodies specific for activated ERK1/2. As shown in Figure 4.12-a, short-term IGF stimulation resulted in phosphorylation of ERK in HB4a cells but not in C3.6 cells, in accordance with the findings by Lu *et al* (Lu *et al.* 2004). Moreover, ERK was phosphorylated only in serum-starved C3.6 cells, probably due to ligand-independent signalling through ErbB2, suggesting that IGF-1-induced ERK phosphorylation is actively inhibited by ErbB2 overexpression. The experiment was repeated with cells also stimulated with HRG and EGF to compare relative levels of ERK activation (Figure 4.12-b). Once

again, ERK phosphorylation in response to IGF-1 was reduced in C3.6 cells. ERK activation was much stronger in response to the ErbB-specific ligands EGF and HRG, particularly in C3.6 cells. Although these results are preliminary and further experiments need to be carried out in order to understand the role of ErbB2 overexpression in IGF signalling, they provide support for the data of Lu *et al.* Interestingly, the same group also reported that increased levels of IGF-1R signalling can confer resistance to Herceptin-induced growth inhibition of breast cancer cell lines, and that addition of IGFBP3 protein to the media could restore the ability of Herceptin to inhibit growth (Lu *et al.* 2001). However, if indeed IGF-1 signalling is abrogated in the C3.6 cell line, the fact that IGFBP3 is downregulated suggests that IGFBP3 does not inhibit IGF-1 signalling in these cells. Nonetheless, IGFBP3 is an interesting ErbB2 target that deserves further investigation, and strategies targeting this pathway may prevent or delay development of Herceptin resistance in ErbB2-overexpressing breast tumours.

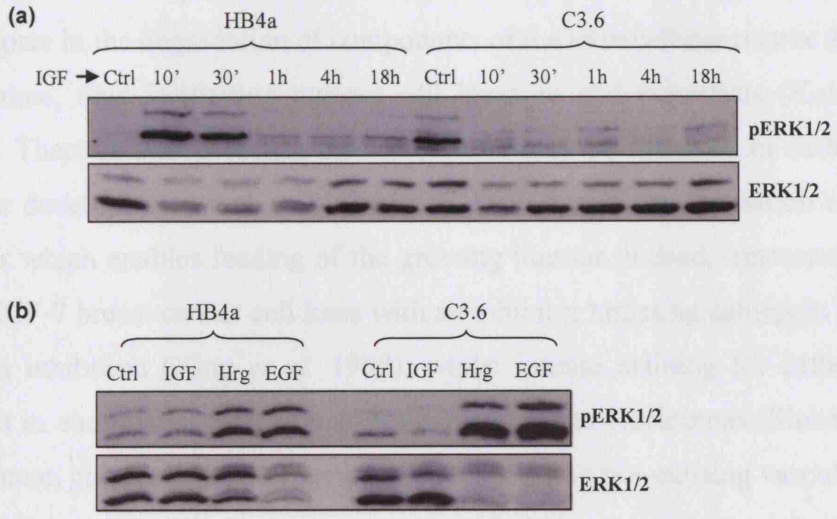

Figure 4.12: Effect of IGF-1 stimulation on MAPK pathway activation in HB4a and C3.6 cells. **(a)** HB4a and C3.6 cells were serum-starved for 24 hours and then stimulated with IGF-1 for the times shown. Activation of the MAPK pathway was analysed using phosphor-ERK1/2 specific antibodies and pan ERK1/2 antibodies. **(b)** HB4a and C3.6 cells were serum-starved for 24 hours and treated with IGF, HRG or EGF for 10 minutes. Activation of MAPK pathway was measure as in (a).

4.3.6 Cathepsins (CTSB & CTSC)

Cathepsin B (CTSB) and Cathepsin C (CTSC) are members of the cathepsin family of ubiquitous lysosomal proteases that are classified both functionally and according to their active site. There are 11 human cathepsins (B, H, L, S, C, K, O, F, V, X and W) and with the exception of cathepsins S, V and K, they are widely expressed in a number of different cells (Turk *et al.* 2001). They are synthesized as inactive precursors, and are activated by proteolytic removal of the N-terminal propeptide by other proteinases or by autocatalytic activation at acidic pH, and are negatively regulated by interaction with their endogenous protease inhibitors, the cystatins (Turk & Bode 1991).

Cathepsins play an important role in many physiological processes such as protein degradation, antigen presentation, bone resorption and hormone processing (Turk *et al.* 2000). They have also been implicated in many pathological processes including cancer progression, particularly cathepsins B, L and D (Fehrenbacher & Jaattela 2005). It has been shown that tumour-associated cathepsins can be translocated to the plasma membrane or secreted from tumour cells, where they participate in the degradation of components of the extracellular matrix and basement membrane, thus facilitating tumour cell invasion and metastasis (Koblinski *et al.* 2000). There is also evidence that cathepsins may be involved in earlier events of tumour development, such as tumour cell proliferation and formation of new blood vessels which enables feeding of the growing tumour. Indeed, treatment of SK-Br-3 and MCF-7 breast cancer cell lines with an inhibitor targeting cathepsin B resulted in growth inhibition (Xing *et al.* 1998), whilst intense staining for cathepsin B was present in endothelial cells of neo-vessels in prostate carcinomas (Sinha *et al.* 1995) and human glioma (Mikkelsen *et al.* 1995), but not in pre-existing vasculature.

Four cathepsin genes were identified in the microarray data analysis discussed here. They were CTSB, CTSC, CTSG and CTSH. Of these, CTSB and CTSC were chosen for qRT-PCR validation because of their interesting pattern of gene expression and response to growth factor stimulation. CTSB is thought to be an important factor in cancer development and progression (see above). However, while CTSB was shown to be involved in the degradation of EGFR and EGF (Authier *et al.* 1999), no reports to date relate the regulation of its expression to ErbB2 or growth factors. On the other hand, the role of CTSC in cancers (if at all) and its expression

regulation are not well documented. Thus, it was of interest to analyse gene expression levels of these cathepsins in the ErbB2 overexpressing cell system and to validate the microarray findings by qRT-PCR.

CTSB was significantly downregulated by HRG in both cell lines as determined by microarray experiments with no changes observed between C3.6 and HB4a cells lines (Figure 4.13-a, left panel). In qRT-PCR experiments however, CTSB was moderately upregulated by EGF and HRG (Figure 4.13-a, right panel). The discrepancy in the results confuses the analysis somewhat and therefore it is not possible to hypothesize the role of ErbB2 or growth factor in the regulation of CTSB expression. Possible reasons for such differences in results obtained between the two platforms are discussed later in this Chapter.

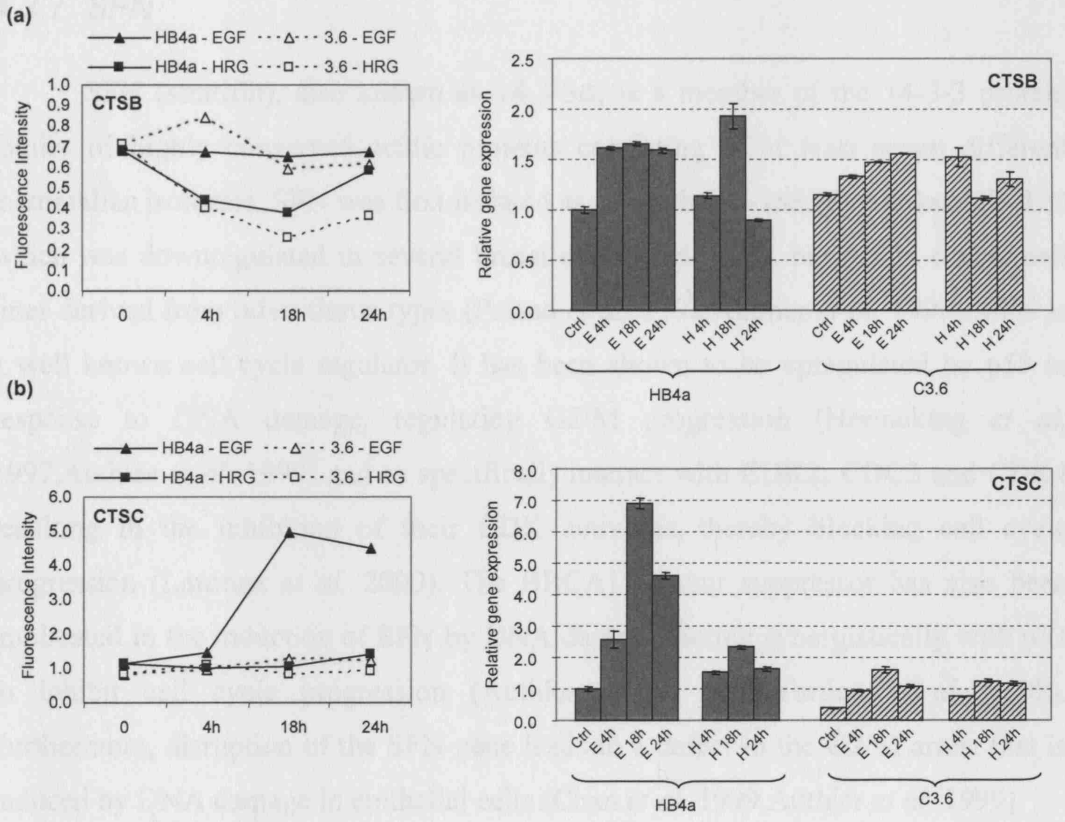

Figure 4.13: Transcriptional regulation of CTSB and CTSC in microarray and qRT-PCR experiments. The left panel represents the microarray data obtained for CTSB (a) and CTSC (b). The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

A much better correlation between microarray and qRT-PCR was observed for the CTSC gene expression (Figure 4.13-b). This gene was highly upregulated by EGF in HB4a cells, and to a lesser extent in C3.6 cells. In microarray experiments, no changes in CTSC levels were observed following HRG stimulation, but a small upregulation was seen in the qRT-PCR results. In addition, CTSC was downregulated basally in C3.6 cells only in the qRT-PCR experiments. These results again suggest that EGF is a more potent regulator of gene expression, and that these effects are more dependent on EGFR levels than ErbB2. The biological significance of these findings are however unclear, and further studies are required to assess if EGF has any effect on CTSC enzyme levels and activity and how this affects cellular function.

4.3.7 SFN

SFN (stratifin), also known as 14-3-3σ, is a member of the 14-3-3 protein family of highly conserved acidic proteins consisting of at least seven different mammalian isoforms. SFN was first isolated as an epithelial-specific marker (HME1) which was downregulated in several breast cancer cell lines, but not in cancer cell lines derived from other tissue types (Prasad *et al.* 1992,Authier *et al.* 1999). SFN is a well known cell cycle regulator. It has been shown to be upregulated by p53 in response to DNA damage, regulating G2/M progression (Hermeking *et al.* 1997,Authier *et al.* 1999) and to specifically interact with CDK2, CDC2 and CDK4 resulting in the inhibition of their CDK activities, thereby blocking cell cycle progression (Laronga *et al.* 2000). The BRCA1 tumour suppressor has also been implicated in the induction of SFN by DNA damage, acting synergistically with p53 to inhibit cell cycle progression (Authier *et al.* 1999,Yarden *et al.* 2002). Furthermore, disruption of the SFN gene leads to a defect in the G2/M arrest that is induced by DNA damage in epithelial cells (Chan *et al.* 1999,Authier *et al.* 1999).

SFN was found to be significantly changing in all conditions in microarray experiments. It was upregulated by EGF, and to a lesser extent by HRG, in both cell lines at the 4 hour time point and was downregulated in C3.6 cells relative to HB4a cells at the 18 and 24 hour time points. This pattern of SFN expression was faithfully reproduced in qRT-PCR experiments (Figure 4.14). The cell line-dependent

difference in SFN expression is probably a reflection of the differential signalling rate between the two cell lines rather than ErbB2 overexpression itself, with SFN returning to basal levels after a shorter time in C3.6 cells compared to HB4a cells following EGF-induced upregulation. This is a novel finding as there are currently no reports on the regulation of SFN gene expression by growth factors. The only indication to date that SFN may be a downstream component of EGF-induced signalling came from proteomics studies in prostate cancer cell lines. SFN was found to become phosphorylated following EGF stimulation, and an increase in SFN protein expression was observed (Authier *et al.* 1999,Huang *et al.* 2004). There are no reports on the role of HRG in the regulation of SFN expression.

SFN expression has been shown to be frequently lost in several types of cancer due to hypermethylation of the gene (Ferguson *et al.* 2000,Iwata *et al.* 2000,Suzuki *et al.* 2000,Osada *et al.* 2002), but no changes in mRNA levels were seen when control C3.6 cells were compared to control HB4a cells. This suggests that although SFN may be a potential marker of breast cancer, its expression is not regulated by ErbB2. Nonetheless, the results presented here indicate that SFN is likely to be a key mediator of EGF- and HRG-induced ErbB signalling.

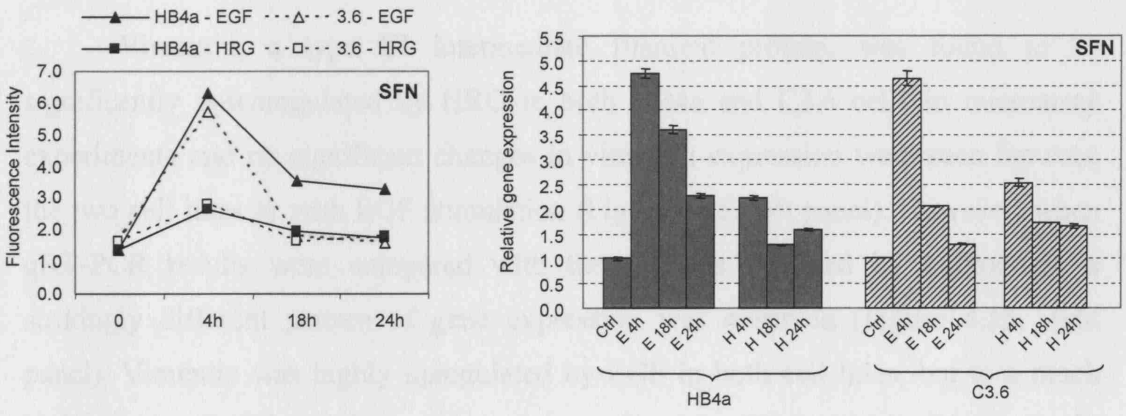

Figure 4.14: Transcriptional regulation of SFN in microarray and qRT-PCR experiments. The left panel represents the microarray fluorescence intensity data for the SFN gene. The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

4.3.8 Adhesion and motility-related genes (ZYX, VIL2 and VIM)

It is thought that successful metastasis formation involves a series of linked sequential steps, requiring tumour cells to dissociate from the primary tumour, invade the surrounding extracellular matrix (ECM), enter the vascular or lymphatic space, escape immune surveillance and mechanical disruption, attach at a distant site, leave the vascular or lymphatic circulation, penetrate the secondary tissue and proliferate as a secondary colony (Chambers *et al.* 2002). Many of these steps require changes in cell shape and motility as well as specific interactions with other cells and the ECM. Thus, cytoskeletal rearrangement and the adhesive interaction between tumour cells and the ECM play a crucial role in metastasis formation. It is well established that aberrant tumour cell adhesion and ECM interactions are causally involved in tumour progression and metastasis (Stetler-Stevenson *et al.* 1993,Gassmann *et al.* 2004). A number of ECM components or genes involved in ECM modelling, degradation and cell adhesion were found to be differentially expressed in one or more conditions studied by microarray (Chapter 3). Three of these, vimentin (VIM), villin 2 (VIL2, also known as ezrin) and zyxin (ZYX) were validated by qRT-PCR and the findings are discussed below.

4.3.8.1 Vimentin

Vimentin, a type III intermediate filament protein, was found to be significantly downregulated by HRG in both HB4a and C3.6 cells in microarray experiments and no significant changes in vimentin expression were seen between the two cell lines or with EGF stimulation (Figure 4.15, left panel). However, when qRT-PCR results were compared with the findings obtained by microarray, a strikingly different pattern of gene expression was observed (Figure 4.15, right panel). Vimentin was highly upregulated by EGF in both cell lines, but to a much higher extent in C3.6 cells, and it was upregulated by HRG only in C3.6 cells. In addition, vimentin levels were almost 5-fold higher in serum-starved C3.6 cells compared to HB4a cells. These data suggest that ErbB2 overexpression and signalling directly control vimentin gene expression, and that this gene was a false positive on the array.

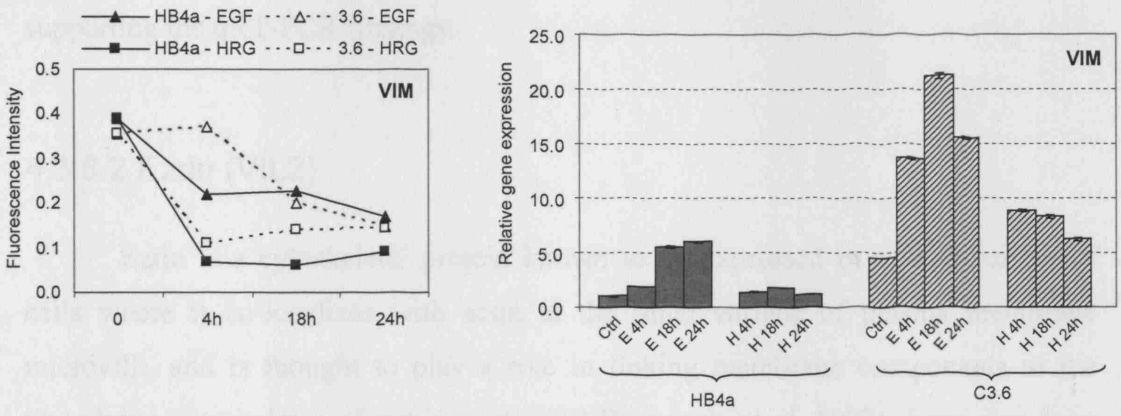

Figure 4.15: Transcriptional regulation of vimentin in microarray and qRT-PCR experiments. Microarray data obtained for the vimentin gene is shown in the left. The results of the qRT-PCR analysis are shown in the right panel. E: EGF, H: HRG.

Although it is difficult to interpret these results because of the striking differences in gene expression patterns, they reinforce the current belief that vimentin plays an important role in breast cancer progression and signalling. Indeed, overexpression of vimentin and its relation to tumour metastasis have been reported in melanoma (Ben Ze'ev & Raz 1985), prostate carcinoma (Lang *et al.* 2002) cervical carcinoma (Gilles *et al.* 1996), hepatocellular carcinoma (Hu *et al.* 2004) and breast cancer (Thompson *et al.* 1992,Sommers *et al.* 1992). Moreover, overexpression of vimentin in a breast carcinoma model lead to increased cell motility and invasiveness *in vitro*, which could be transiently down-regulated by treatment with antisense oligonucleotides to vimentin (Hendrix *et al.* 1997). It is therefore likely that the upregulation of vimentin in C3.6 cells seen in qRT-PCR experiments represents a more accurate reflection of the pattern of expression of vimentin in these cells. Indeed, vimentin was also found to be upregulated in C3.6 cells in the microarray experiments performed by White *et al* and Mackay *et al* (Mackay *et al.* 2003,White *et al.* 2004), further reinforcing the hypothesis that ErbB2 positively regulates the expression of vimentin. The role of growth factor regulation of vimentin gene expression is less clear. There are no reports to date on the role of HRG in regulating vimentin expression, although EGF increased its expression in breast carcinoma cells,

and this was followed by increased motility of these cells (Ackland *et al.* 2003), supporting the qRT-PCR findings.

4.3.8.2 Ezrin (VIL2)

Ezrin is a cytoskeletal protein known to be expressed in a wide variety of cells where it co-localizes with actin at the inner surface of plasma membrane microvilli, and is thought to play a role in linking membrane components to the underlying cytoskeleton (Franck *et al.* 1993,Berryman *et al.* 1993). Ezrin has been shown to play a positive role in maintaining cell shape and cell polarity and participates in membrane-trafficking pathways, cell migration, cell signalling, growth regulation, and differentiation (Tsukita & Yonemura 1997). Ezrin has also been proposed to be actively involved in tumour biology, especially in regulating the growth and metastatic capacity of cancer cells, with overexpression of ezrin detected in several human epithelial tumours, including brain hemangioblastoma (Bohling *et al.* 1996), uterine endometrioid adenocarcinoma (Ohtani *et al.* 2002), osteosarcoma (Khanna *et al.* 2001) and uveal malignant melanoma (Makitie *et al.* 2001).

In both microarray and qRT-PCR experiments, ezrin was found to be transiently upregulated by EGF in both HB4a and C3.6 cells lines and by HRG only in the C3.6 cells (Figure 4.16). In qRT-PCR experiments, the induction of ezrin by HRG in C3.6 cells was as potent as its induction by EGF and its basal levels were higher. As discussed in Chapter 3, ezrin is one of a number of genes that are responsive to HRG only in the C3.6 cells. These genes are interesting because they are likely to contribute to abnormal and/or enhanced signalling in ErbB2-overexpressing breast cancers and thereby play a role in altering cell properties and rendering them more prone to transformation. It is likely that such an effect is associated with the higher levels of ErbB3 observed in the C3.6 cells, and therefore signalling through the potent ErbB2/ErbB3 receptor dimer.

In addition, by linking the membrane and the actin cytoskeleton, ezrin is thought to participate in signal transduction. Ezrin is a known substrate for phosphorylation by EGFR, and its phosphorylation kinetics following EGF stimulation parallels EGF-induced morphological changes (Bretscher 1989). Thus, ezrin phosphorylation may play a role in regulating cell surface cytoskeletal

rearrangement in response to EGF. Phosphorylation of residues Y145 and Y353 were detected to high stoichiometry after EGF treatment of human epithelial-derived A431 cells (Krieg & Hunter 1992). Phosphorylation of ezrin at Y353 has been shown to be required to activate survival signals during epithelial cell differentiation via the PI3K/Akt pathway (Gautreau *et al.* 1999), whereas ezrin Y145 phosphorylation was shown to occur following binding to Src and be involved in mediating signalling events leading to epithelial cell spreading and proliferation (Srivastava *et al.* 2005). The results shown here suggest that, in addition to phosphorylating ezrin, EGF acts at the transcriptional level to upregulate ezrin mRNA levels in cells, and also suggest that HRG may play a role in its regulation in ErbB2-overexpressing cells. Whether the aberrant regulation of ezrin is a relevant factor for invasiveness of breast cancer awaits additional studies.

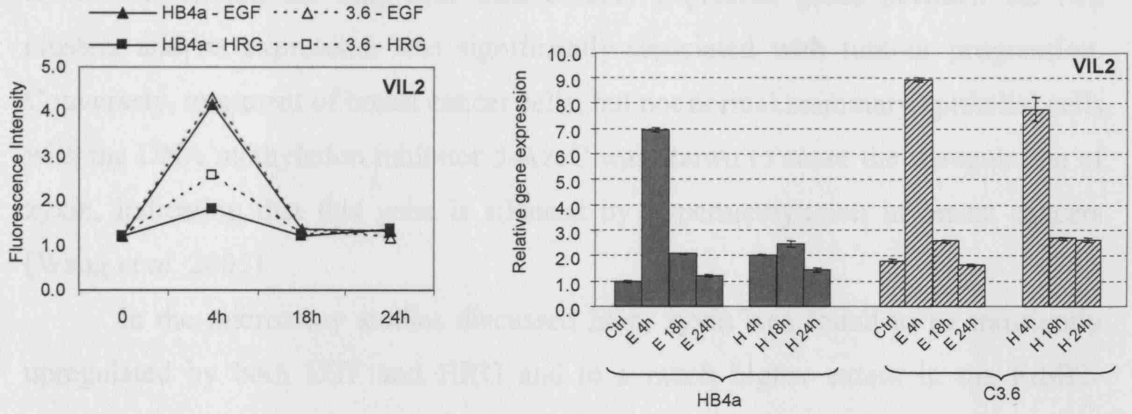

Figure 4.16: Transcriptional regulation of ezrin (VIL2) in microarray and qRT-PCR experiments. Microarray data obtained for the ezrin gene is shown in the left. The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

4.3.8.3 Zyxin (ZYX)

Zyxin is a zinc-binding phosphoprotein that co-localizes with the actin cytoskeleton at sites where the fast-growing ends of actin filaments are enriched, including lamellipodia and focal adhesions, where it is proposed to dock proteins involved in cytoskeletal organization and dynamics (Beckerle 1997). Zyxin has been shown to be an important regulator of cell spreading and migration by acting as a molecular scaffold, facilitating the formation of a molecular complex that promotes site-specific actin assembly (Reinhard *et al.* 1999).

Changes in zyxin expression have been implicated in altered cancer cell growth and behaviour, although the exact role of zyxin in cancer development is not understood. Increased zyxin expression was observed in melanoma cells compared to their biological precursor cells (melanocytes), and its expression correlated with cell spreading and proliferation (van der Gaag *et al.* 2002). Microarray gene expression analysis of nine bladder cancer cell lines resulted in the formation of two major clusters based on their p53, pRb and INK4A status (Sanchez-Carbayo *et al.* 2002). Zyxin was among the only four differentially expressed genes between the two clusters and its expression was significantly associated with tumour progression. Conversely, treatment of breast cancer cells, but not normal mammary epithelial cells, with the DNA methylation inhibitor 5-AzaC was shown to cause the upregulation of zyxin, indicating that this gene is silenced by hypermethylation in breast cancers (Wang *et al.* 2005).

In the microarray studies discussed here, zyxin was found to be transiently upregulated by both EGF and HRG and to a much higher extent in the ErbB2-overexpressing cells, and therefore was placed in the list of genes changing in response to all experimental conditions (Figure 4.17, left panel). Together with the findings discussed above, these data suggest that zyxin may play a key role in cancer development and progression, and therefore was chosen for further validation by qRT-PCR. The expression pattern of zyxin found in microarray experiments was confirmed in qRT-PCR experiments (Figure 4.17, right panel). Zyxin was transiently upregulated by EGF in both cell lines, but to a much higher extent in C3.6 cells, and by HRG only in C3.6 cells. In addition, zyxin was 4-fold higher in serum-starved C3.6 cells compared to control HB4a cells. These findings indicate that zyxin is a target of ErbB2 overexpression and downstream signalling and is therefore

implicated in ErbB2-dependent transformation through its effects on cell shape and motility.

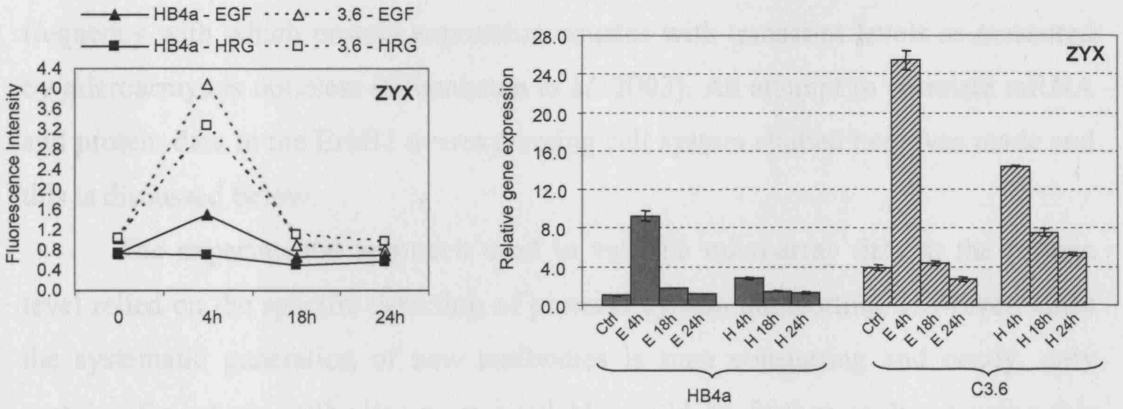

Figure 4.17: Transcriptional regulation of zyxin (ZYX) in microarray and qRT-PCR experiments. Microarray data obtained for the zyxin gene is shown in the left. The validation of these results by qRT-PCR is shown in the right panel. E: EGF, H: HRG.

It is interesting to note that the pattern of gene expression between zyxin and ezrin are very similar, although the extent of mRNA upregulation was not identical. Both these proteins, as well as VASP, another phosphoprotein associated with actin filament and focal contact areas, were found to be enriched at sites of Cdc42-induced actin polymerization, suggesting that they act in conjunction to create membrane protrusions necessary for cell migration (Castellano *et al.* 1999). VASP was also identified here by microarray analysis as an EGF-responsive gene, and its expression pattern was similar to that of zyxin and ezrin. It is therefore possible that these genes, and possibly others not identified in the present study, are co-regulated downstream of ErbB receptor signalling in breast cancers to induce a more motile cellular phenotype that is susceptible to metastasis.

4.4 Comparison of mRNA and Protein Expression

In addition to validating results at the mRNA level, it is equally important to evaluate expression levels of the corresponding protein products. Combining both gene expression and protein expression data can provide important information concerning protein post-transcriptional regulation and function. At present, the frequency with which protein expression equates with transcript levels as measured by microarrays is not clear (Greenbaum *et al.* 2003). An attempt to correlate mRNA and protein data in the ErbB2 overexpressing cell system studied here was made and this is discussed below.

The experimental approach used to validate microarray data at the protein level relied on the specific detection of proteins by immunoblotting. However, since the systematic generation of new antibodies is time consuming and costly, only proteins for which antibodies were available could be further analysed using this method. Total cellular extracts were analysed using a panel of antibodies specific for the proteins of interest (Table 2.2, Chapter 2). In each western blotting experiment carried out, cdk4 was used as a loading control as it was shown that in all conditions tested, levels of cdk4 remain unchanged (data not shown). Antibody specificity was confirmed by the fact that discrete bands were detected at the expected molecular weight.

Of the genes validated by qRT-PCR, antibodies were obtained for p48 and IGFBP3. The mRNA for both genes was found to be downregulated in C3.6 cells by microarray as well as qRT-PCR. This downregulation was confirmed at the protein level (Figure 4.18), suggesting that, at least for these genes, differences in gene expression are reflected at the protein level. Thus, ErbB2 overexpression may affect the levels of these proteins within cells to alter their physiological responses and change their proliferative behaviour. IGFBP3 was identified as a doublet of 44 and 41KDa due to different glycosylation states, as previously demonstrated (Firth & Baxter 1995,Bhat *et al.* 1997). Moreover, while IGFBP3 mRNA was significantly downregulated by EGF, no changes in protein levels were observed following growth factor stimulation. These findings support the notion that IGFBP3 can be regulated at many levels. Indeed, IGFBP3 has been previously shown to be regulated both transcriptionally by p53-dependent and independent pathways (Buckbinder *et al.* 1995,Choi *et al.* 2002) and post-translationally by modifications which include glycosylation, phosphorylation, proteolysis, and cell-membrane and ECM

association (Conover 1996). Thus, while EGF can downregulate IGFBP3 mRNA expression, its protein levels remain constant and may be subject to other modes of regulation under different conditions.

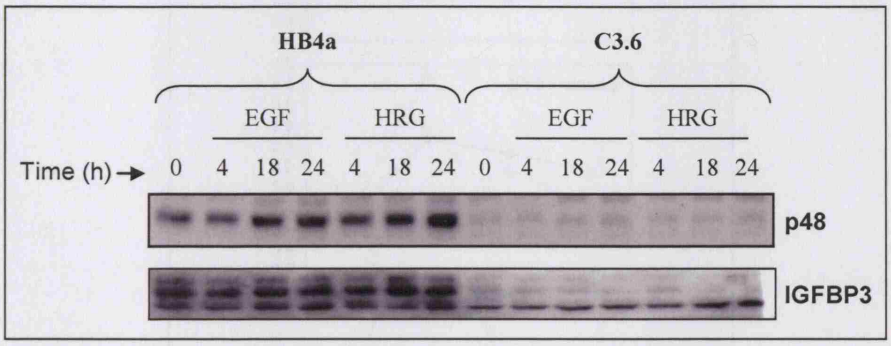

Figure 4.18: Validation of p48 and IGFBP3 microarray expression data by western blotting. HB4a and C3.6 cells were serum-starved for 48 hours and then stimulated with EGF and HRG for the same time points studied by microarrays. Cell lysates were subjected to western blotting using p48- or IGFBP3-specific antibodies.

The proto-oncogene Myc was represented twice on the array, and both clones showed a similar pattern of gene expression (Figure 4.19). Myc mRNA expression was not different between HB4a and C3.6 cells under serum-starved conditions. However, it was significantly upregulated following 4 hour stimulation by EGF and to a higher extent in C3.6 cells, and was upregulated by HRG only in C3.6 cells. Western blotting for Myc was carried out previously in our laboratory (Timms *et al.* 2002) and showed an identical pattern of protein expression at the 4 hour time point (Figure 4.19). These results suggest that ErbB2 overexpression directly affects Myc protein levels, and that the expression of Myc protein in response to growth factors is transcriptionally regulated.

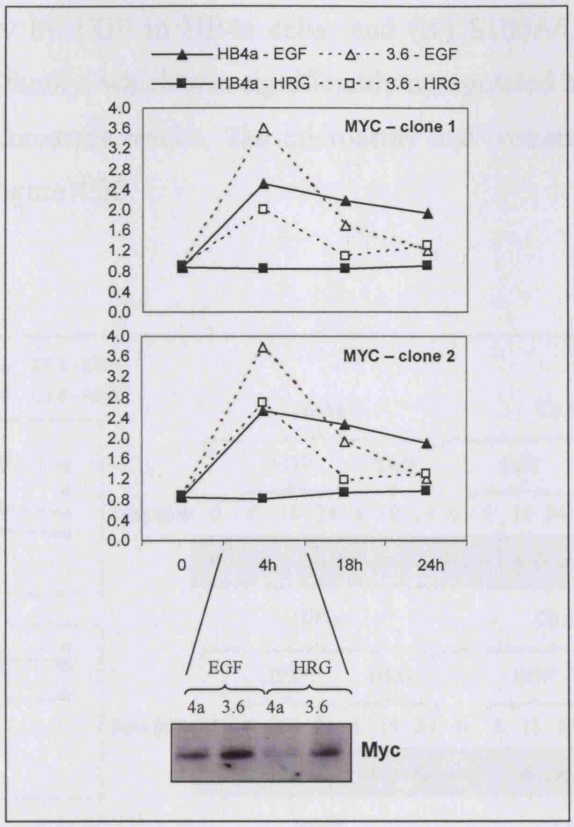

Figure 4.19: Validation of Myc regulation by growth factor stimulation. Two Myc clones were represented on the array, both showing strikingly similar pattern of gene expression. Myc western blotting, shown here only for the 4 hour time point stimulation, was carried out by Timms et al (Timms *et al.* 2002).

Western blotting validation of microarray data was also carried out for the following genes: (i) prohibitin (PHB), an anti-proliferative gene thought to bind pRb and modulate the activity of E2F transcription factors (Wang *et al.* 1999). PHB was significantly upregulated in C3.6 cells and also following EGF and HRG stimulation in microarray experiments; (ii) peroxiredoxin 1 (PRDX1), an antioxidant enzyme which functions as a regulator of H_2O_2 levels as well as an intracellular signalling molecule in many cytokine-induced signal transduction pathways (Wood *et al.* 2003). PRDX1 was upregulated by both EGF and HRG in C3.6 cells, and by EGF alone in HB4a cells; (iii) dual specificity phosphatase 1 (DUSP1 or MKP1), which regulate the phosphorylation status of MAP kinases by removing phosphate groups from both tyrosine and serine/threonine residues (Theodosiou & Ashworth 2002). In the microarray experiments, DUSP1 was transiently upregulated by EGF and HRG in

C3.6 cells, but only by EGF in HB4a cells; and (iv) S100A6, a calcium binding protein of the S100 family, which was significantly upregulated by EGF in HB4a and C3.6 cells in the microarray results. The microarray and western blot data for these genes is shown in Figure 4.20.

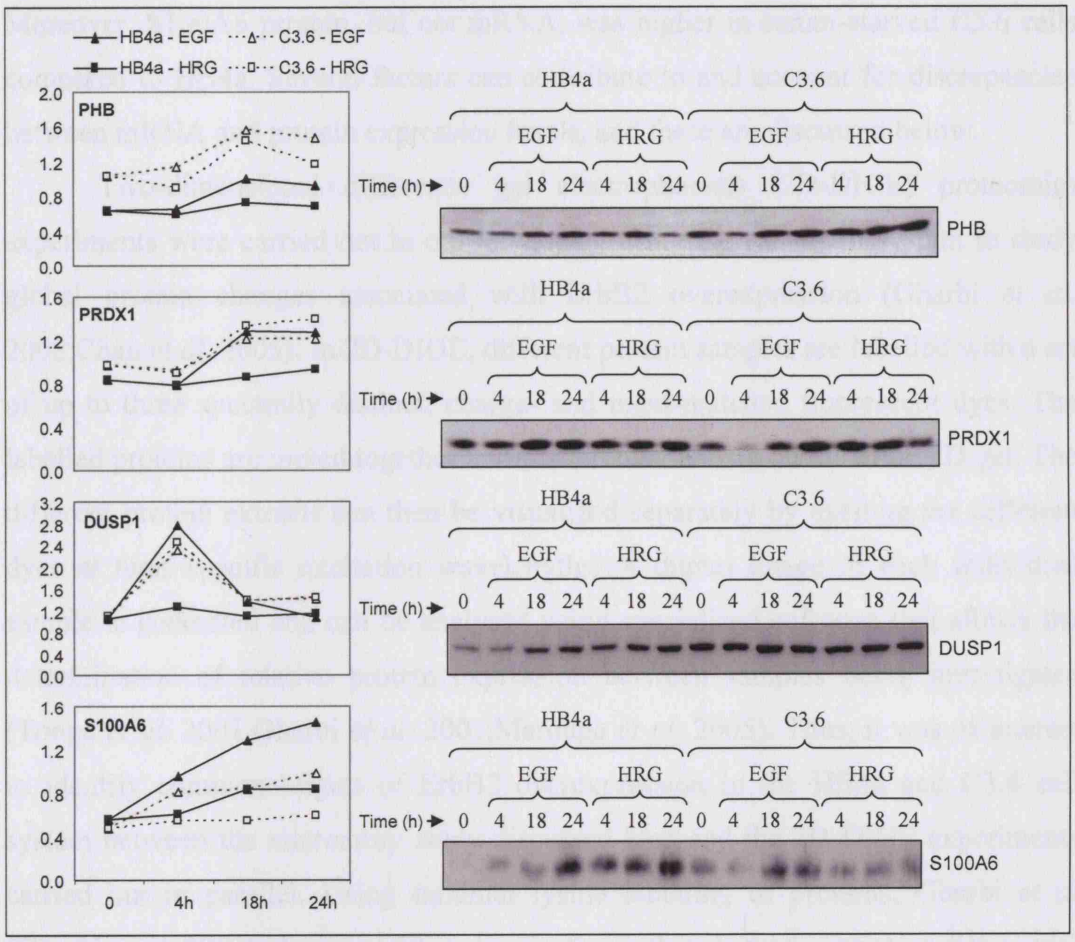

Figure 4.20: Western blotting validation of microarray expression data for PHB, PRDX1, DUSP1 and S100A6. Cell lysates were prepared as described in Figure 4.18 and immunoblotting was carried out using antibodies specific for PHB, PRDX1, DUSP1 and S100A6.

While some similarities in expression changes between mRNA and protein were found, the correlation for these genes was generally poor. PHB was upregulated in C3.6 cells compared to HB4a cells in the immunoblots, and a mild upregulation by following growth factor stimulation can be seen. However, the growth factor-induced

changes in gene expression seen in microarray experiments for PRDX1 were not reproduced at the protein level, although these changes were small. DUSP1 protein showed a general upregulation in C3.6 cells, in contrast to the transient upregulation induced by EGF and HRG in microarray experiments. The best correlation was for S100A6 protein, which was upregulated in a time-dependent manner by both EGF and HRG in HB4a cells, but only by EGF in C3.6 cells in both experiments. Moreover, S100A6 protein, but not mRNA, was higher in serum-starved C3.6 cells compared to HB4a. Several factors can contribute to and account for discrepancies between mRNA and protein expression levels, and these are discussed below.

Two-dimensional difference gel electrophoresis (2D-DIGE) proteomics experiments were carried out in our laboratory using the same cell system to study global protein changes associated with ErbB2 overexpression (Gharbi *et al.* 2002,Chan *et al.* 2005). In 2D-DIGE, different protein samples are labelled with a set of up to three spectrally distinct, charge- and mass-matched fluorescent dyes. The labelled proteins are mixed together and run simultaneously on the same 2D gel. The different protein extracts can then be visualized separately by exciting the different dyes at their specific excitation wavelengths. A digital image of each individual sample is generated and can be analysed using specialized software that allows the determination of relative protein expression between samples being investigated (Tonge *et al.* 2001,Gharbi *et al.* 2002,Marouga *et al.* 2005). Thus, it was of interest to identify common targets of ErbB2 overexpression in the HB4a and C3.6 cell system between the microarray study discussed here and the 2D-DIGE experiments carried out in parallel. Using minimal lysine labelling of proteins, Gharbi *et al* (Gharbi *et al.* 2002) identified 30 protein isoforms that showed consistent differential expression between HB4a and C3.6. Chan *et al* (Chan *et al.* 2005) used a high stoichiometry cysteine labelling approach and identified 26 gene products that were differentially expressed between HB4a and C3.6 cells. A number of proteins identified in these two studies were also identified in the microarray study described here. Seven genes were upregulated in both microarray and proteomics studies. These were the hydrolase HIBCH, L-plastin (LCP1), the aldose reductase AKR1B1, copine 3 (CPNE3), nucleoside diphosphate kinase A (NME1), keratin 13 and the proteinase inhibitor SERPINE1. Downregulated genes and proteins were FK506 binding protein 4 (FKBP4), glutathione S-transferase π (GSTP1) and the ubiquitin-specific protease 14 (USP14). Both SFN and ezrin were significantly downregulated

in the proteomics analysis, but showed no difference in mRNA expression levels in C3.6 cells compared to HB4a in either the microarray or the qRT-PCR experiments (Figure 4.14 and Figure 4.16), suggesting that these genes may be affected by ErbB2 post-transcriptionally despite being upregulated at the mRNA level by growth factor stimulation. Another three genes – heat shock protein 27 (HSPB1), ubiquitin-conjugating enzyme E2N (UBE2N) and elfin (PDLIM1) – were differentially regulated between the two cell lines in the proteomics studies, but their mRNA expression was changing significantly only in response to growth factor stimulation and not between cell lines.

The mRNA expression data from White *et al* (White *et al.* 2004) was compared with the protein expression ratios from 2D-DIGE analysis (Gharbi *et al.* 2002) and from previous western blotting analyses ((Timms *et al.* 2002) and unpublished data) obtained by densitometry of films, representing a total of 43 genes which were present on the microarray chips. A statistically significant relationship between protein ratios and mRNA ratios was observed with a correlation coefficient of 0.81 (p <0.001) and a linear regression of 0.66 (p <0.001) (White *et al.* 2004). This high correlation shows that a large number of these proteins are transcriptionally regulated. However, the mRNA and protein data did not correlate for all genes analysed, for example EGFR and YWHAB (14-3-3β), suggesting that these genes may be regulated post-transcriptionally. Many of the genes used in this mRNA and protein correlation analysis were also present in the microarray study described here. These include copine 3, ISGF3G (p48), ErbB2, ErbB3, HIBCH, and LCP1. Thus, although mRNA levels are not direct predictors of a gene's protein levels, combining both gene expression and protein expression data can provide important information concerning protein post-transcriptional regulation and function.

4.5 Chapter Conclusions & Discussion

DNA microarray technology is a powerful technique that allows the measurement of the expression of thousands of genes simultaneously. However, this technology is prone to errors and variation that can result from both biological as well as technical sources. Thus, validating the results obtained from microarray studies is crucial when trying to understand the molecular events associated with the

system being studied. An evaluation of the application of microarray technology for the expression profiling of a model cell system to study the effects of ErbB2 overexpression has been investigated in this Chapter. The reliability of this method was tested by data validation using quantitative real-time PCR analysis and western blotting.

Fourteen genes identified from the microarray study described in Chapter 3 were chosen for validation by qRT-PCR. With the exception of vimentin and cathepsin B, a high correlation between qRT-PCR and microarray data was obtained, indicating that this is a useful strategy to validate microarray data. All 12 genes were validated both with regard to their differential expression associated with high ErbB2 levels as well as in their response to growth factor stimulation. Note that although the directionality of changes was similar between the two platforms, the fold-changes observed were several-fold higher in qRT-PCR experiments. Possible explanations for these findings are discussed in Chapter 6. In addition to the 14 genes validated by qRT-PCR that were significantly changing in the microarray experiment, three IFN-stimulated genes, which were expected to be downregulated in C3.6 cells but were not identified by microarray analysis, were analysed by qRT-PCR. All of them were indeed found to have lower mRNA levels in C3.6 cells compared to HB4a cells. This highlights the increased sensitivity and reproducibility of the qRT-PCR method compared to microarray.

One possibility for the discrepancies between the two platforms is that the non-verifiable genes may represent splice variants. Because the genome is still imperfectly annotated, the cDNA probes on the arrays may target one or more variants, which may be different from the variant amplified by PCR. Thus, if these splice variants are indeed differentially expressed relative to one another, the two platforms will measure different expression patterns. Indeed, cathepsin B has been shown to give rise to mRNA splice variants that are differentially expressed in human tumours (Gong *et al.* 1993). An alternative explanation for the discordance between the two platforms is that gene family members may cross-hybridize with the cDNA probes on the array whereas qRT-PCR is probably more specific and amplifies just one member. Regardless of the reason for the discordance between microarray and qRT-PCR data for vimentin and cathepsin B, it is not possible at present to hypothesize whether the mRNA expression of these genes is regulated by ErbB2 and/or the growth factors EGF and HRG.

In addition to validating microarray data by qRT-PCR, a comparison between mRNA and protein data was attempted. Constant effort is being applied to combine the complex data generated from both proteomic analyses and microarray technology (Gygi *et al.* 1999,Chen *et al.* 2002,Patel *et al.* 2002,White *et al.* 2004). Western blotting experiments confirmed the downregulation of the IFN-stimulated gene p48 and of IGFBP3, and the upregulation of prohibitin in serum-starved C3.6 cells compared to HB4a. A poorer correlation was observed for the other genes studied, where DUSP1 and S100A6 appeared to be upregulated in C3.6 cells at the protein level and not mRNA. In addition, only S100A6 could be upregulated by growth factors in both microarray and immunoblot experiments. All other genes showed no similarities in their mRNA and protein expression profiles following EGF or HRG stimulation. Although this data appears to be contradictory, the rate of protein turn-over in both cell lines needs to be further investigated, as this could affect the results. It is possible that, while ErbB2 overexpression can regulate changes in gene expression that are reflected at the protein level, growth factors can alter mRNA expression levels, but this may not necessarily result in protein level changes. In addition to the western blotting experiments, microarray data was compared with parallel proteomics experiments carried out in our laboratory. In these studies, proteins that were differentially expressed in un-stimulated C3.6 cells compared to HB4a cells were identified (Gharbi *et al.* 2002,Chan *et al.* 2005). A number of genes in the microarray experiment were indeed also identified in the proteomics analysis, suggesting that their expression is transcriptionally regulated by ErbB2.

Once microarray data has been analysed and independently verified, the next step is to investigate whether the identified targets do indeed represent a biologically significant target. This can be done by assessing a gene set that is considered critical in a larger and more extensive study group. Tissue microarrays (TMA) are an excellent approach for the validation of array data in larger sets of human or animal tissues. TMAs are collections of hundreds of tissue slices from cores arrayed onto slides, allowing investigators to conduct controlled studies on large cohorts of tissues and therefore obtain enough statistical power for meaningful analysis, including *in situ* detection of target DNA, RNA and/or protein expression data from biological replicates (Kononen *et al.* 1998). Many researchers have used TMAs in combination with DNA microarray to validate targets discovered by gene expression profiling. One of the earliest and most comprehensive studies using these two technologies in

combination was the work of Dhanasekaran *et al* (Dhanasekaran *et al.* 2001a). These authors analysed 50 normal and cancerous prostate specimens by cDNA microarray and then further validated two of the genes differentially expressed (hepsin, a transmembrane serine protease, and pim-1, a serine/threonine kinase) using TMAs consisting of over 700 prostate cancer specimens. These two genes were found to be significantly correlated with measures of clinical outcome. Thus, the combination of cDNA and tissue microarray technologies can enable rapid identification of genes associated with progression of human cancers and may facilitate analysis of the role of the encoded gene products in the pathogenesis of this disease. We therefore decided to carry out immunohistochemistry analysis of the IFN-inducible gene p48 on a TMA containing 257 breast cancer specimens. This work was carried out in collaboration with Prof. Sunil R. Lakhani (Institute for Cancer Research, UK, at the time of collaborative studies, presently at the Queensland Institute of Medical Research, Australia). Only 71 (28%) of the tumour samples showed strong p48 expression, and p48 expression did not correlate significantly with prognostic factors such as tumour grade, vascular invasion, lymph node metastasis or overall survival. However, statistical analysis to examine associations between p48 antibody staining in ErbB2-positive or negative tumour groups was not carried out. This work was therefore inconclusive with respect to the role of ErbB2 overexpression in the regulation of p48 levels in breast cancer. Interestingly, tumours positive for p48 tended to be oestrogen receptor (ER)-positive (p=0.0038). Patients with ErbB2-overexpressing breast carcinomas do not respond to tamoxifen (hormone) therapy (Houston *et al.* 1999), and ER expression has been previously shown to be generally inversely correlated with ErbB2 expression in breast cancers (Lal *et al.* 2005). The importance of these findings are not known at this time, but they point to a role for p48 in breast cancer therapy response. Regardless of the TMA results, the downregulation of IFN-stimulated genes in the ErbB2-overexpressing breast cancer cells was considered to be a significant and novel finding, and further validation of the IFN signalling pathway was carried out in order to try and establish the biological mechanisms of this pathway in breast cancer. This work is described in Chapter 5.

In conclusion, qRT-PCR is a reliable method to validate microarray data and identify interesting targets of ErbB2 overexpression. The data presented here showed that the altered phenotype (morphology, proliferation and adhesion) observed between HB4a and C3.6 may be represented by the genes found to be differentially

expressed in the microarray analysis, and that changes in the expression of important genes involved in these processes could be confirmed by qRT-PCR. In addition, combining microarray analysis with data obtained by proteomics-based approaches enabled improved understanding of the regulatory network downstream of ErbB2 overexpression. This work has shown that, in the breast cancer system studied here, protein abundance is likely to be a reflection of the altered transcription of a subset of genes, but translation and post-translational modifications also appear to influence the expression levels of many individual proteins in ErbB2-overexpressing breast cancers.

Chapter 5: ERBB2-DEPENDENT SUPPRESSION OF INTERFERON SIGNALLING

5.1 Chapter Introduction

Chapter 3 showed the power of microarray technology for comprehensive gene expression profiling in the study of ErbB2-dependent cell transformation and growth factor signalling through the ErbB receptor family. However, such experiments do not provide sufficient information to allow a conclusion on what the biological roles of the identified targets might be. Even in time course experiments, where temporal information on gene expression patterns is available, it is impossible to determine the downstream interactions and pathways leading to a particular response without further experiments. This Chapter addresses this by further analysing the level of interaction between the ErbB and interferon (IFN) signalling pathways. A number of IFN-stimulated genes were found to be differentially regulated in ErbB2 overexpressing cells and therefore could provide a potential link between ErbB2 overexpression and de-regulated proliferation of cancer cells. A variety of traditional biochemical techniques were therefore used to follow up on the findings of the microarray analysis.

5.2 Introduction to the IFN Signalling Pathway

Interferons (IFNs) are a family of related cytokines that were originally identified as the proteins responsible for the induction of cellular resistance to viral infection. Subsequently, much evidence has been accumulated indicating that IFNs mediate a range of diverse functions including the (negative) regulation of cell proliferation, differentiation, apoptosis and immunomodulation. There are two major types of IFNs: type I, composed of IFNα, IFNβ, IFNω, IFNτ and IFNκ, which are produced by a variety of cells upon viral infection; and type II, or IFNγ, which is produced by activated T cells and Natural Killer (NK) cells (Stark *et al.* 1998). Following secretion from cells, IFNs mediate their effects by binding to type I or type II-specific cell surface receptors. These receptors lack intrinsic tyrosine kinase activity and therefore rely on the Janus Kinases (JAKs), a family of receptor-

associated cytoplasmic proteins currently composed of JAK1, JAK2, JAK3 and TYK2 (Aaronson & Horvath 2002). JAKs catalyse ligand-induced self-phosphorylation and also the phosphorylation and activation of the signal transducers and activator of transcription (STAT) family of transcription factors. STAT proteins then homo- or heterodimerize and can form complexes with other transcription factors to activate transcription of IFN-stimulated genes (ISGs). These gene products regulated by IFNs are the primary effectors of the IFN-mediated biological responses (Darnell, Jr. *et al.* 1994,Stark *et al.* 1998).

Figure 5.1 shows a schematic representation of type I and II IFN signalling pathways. Type II IFN signalling begins with IFNγ binding to IFNG receptor-1. This leads to the association of IFNG receptor-2 to the complex, bringing receptor-associated JAKs into proximity for cross-phosphorylation and activation of JAKs and of the receptors themselves. This creates docking sites for STAT1, which is phosphorylated, homodimerizes and translocates to the nucleus. STAT1 homodimers bind to specific gamma-activated site (GAS) elements of IFN-inducible genes to stimulate their transcription. (Boehm *et al.* 1997). IFNα and IFNβ, on the other hand, induce IFNA receptor-1 and -2 dimerization and phosphorylation of STAT1 and STAT2 through the kinase activity of TYK2 and JAK1. STAT1 and STAT2 then form an oligomeric complex in the nucleus called ISGF3, which also contains a third protein, the DNA-binding adapter protein p48 (also known as ISGF3γ or IRF9). ISGF3 induces transcription of IFN-stimulated genes by binding to so-called IFN-stimulated response elements (ISRE) (Brierley & Fish 2002). Although these direct transcriptional activation events do not require the synthesis of new transcription factors, several primary response genes are themselves transcription factors and are required for the induction of other secondary components of the cellular response to IFNs. A major difference between GAS elements and the ISRE sites is that the former binds dimerized STAT proteins, whereas the latter are targeted by members of the IFN regulatory factor (IRF) family, either alone or in conjunction with associated proteins (Bluyssen *et al.* 1996,Levy 1998). Although these two signalling systems were previously thought to be independent, it is now recognized that there is considerable crosstalk between type I and type II IFN signalling, where the ISRE has been found to bind factors in response to all IFN types (IFNα, IFNβ and IFNγ), and GAS elements can bind proteins activated by both type I and type II IFNs.

Furthermore, GAS elements bind proteins activated by a wide variety of cytokines and growth factors in addition to IFNs (Darnell, Jr. 1997). Finally, autocrine amplification of IFN signalling is achieved through IFN-induced IFN expression and secretion from cells. Both ISGF3 and IRF7, which is itself induced by type I IFNs, have been shown to activate the transcription of both IFNα and IFNβ (Yoneyama *et al.* 1996,Levy *et al.* 2002).

Oligonucleotide microarray studies in melanoma (Leaman *et al.* 2003), fibrosarcoma (Der *et al.* 1998) and in IFN-treated mouse embryonic and human dendritic cells (de Veer *et al.* 2001) have identified over 300 IFN-induced genes, making it difficult to attribute the effects of IFNs to any particular gene product. However, the best characterized IFN-stimulated genes are the ones that play a role in the anti-viral response, such as double stranded RNA (dsRNA)-dependent protein kinase (PKR) and 2'-5'-oligoadenylate synthetase (OAS), which inhibit protein synthesis and promote viral RNA cleavage, respectively. The Mx proteins are also induced by IFNs and are known to interfere with viral replication (Stark *et al.* 1998).

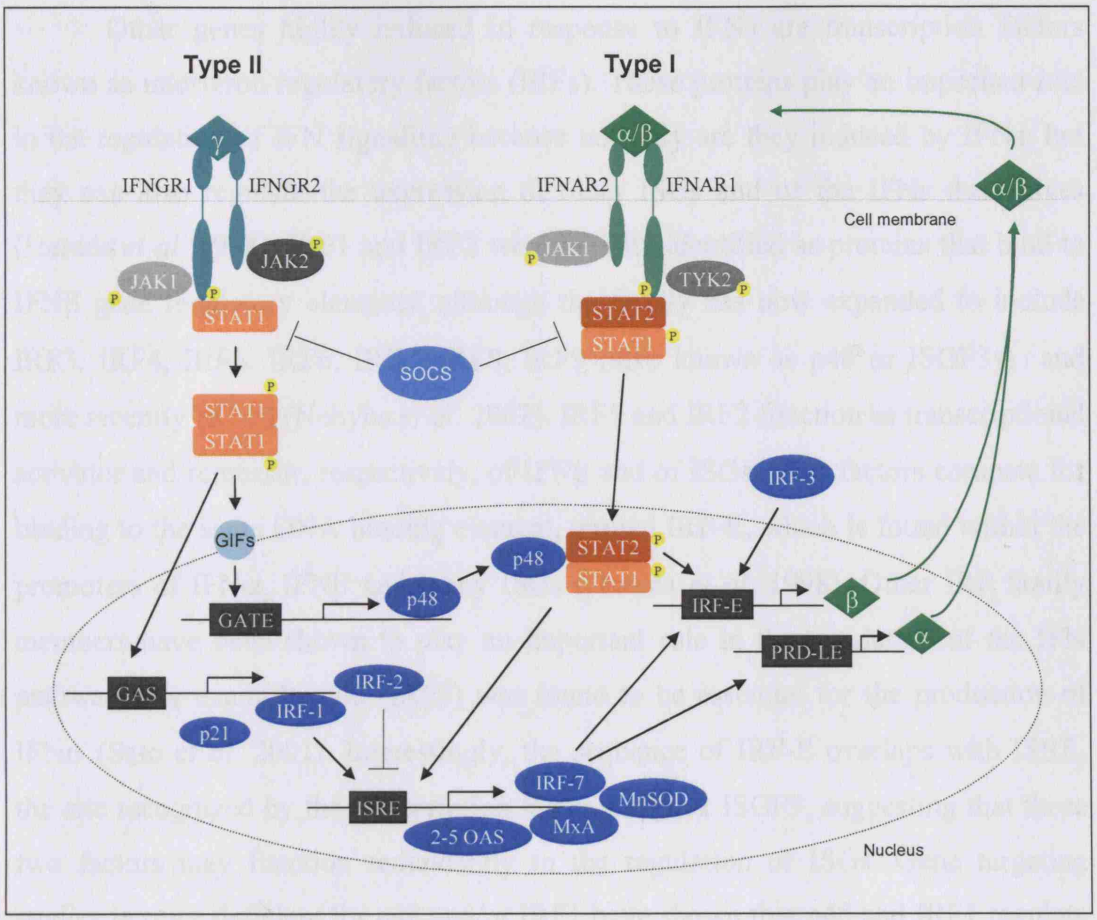

Figure 5.1: The IFN signalling pathway. Type I and Type II IFNs signal through different receptors, activating different JAKs and STAT dimers and leading to the transcription of various IFN-stimulated genes (ISGs, represented in blue). Although each IFN activates genes with distinct sequence elements in their promoters (ISRE sequence for Type I and GAS sequence for Type II), there is a degree of overlap between the two types of IFN signalling, where some genes can be regulated directly or indirectly by both types of IFNs. Note that the expression of p48 is induced by IFNγ through the IFNγ-activated transcriptional element (GATE) on the p48 promoter, and this has been shown to be mediated by proteins termines GIFs (Weihua *et al.* 1997a). IFN signalling can be also potentiated through a positive feedback loop, whereby IFN itself can be produced in response to IFN stimulation. Negative regulation of IFN signalling can be achieved through SOCS proteins, which are thought to inhibit JAK catalytic activity and/or block access of STATs to the receptor binding site (Kile & Alexander 2001).

Other genes highly induced in response to IFNs are transcription factors known as interferon regulatory factors (IRFs). These proteins play an important role in the regulation of IFN signalling because not only are they induced by IFNs, but they can also regulate the expression of other ISGs and of the IFNs themselves (Harada *et al.* 1998). IRF1 and IRF2 were initially identified as proteins that bind to IFNβ gene regulatory elements, although the family has now expanded to include IRF3, IRF4, IRF5, IRF6, IRF7, IRF8, IRF9 (also known as p48 or ISGF3γ) and more recently IRF10 (Nehyba *et al.* 2002). IRF1 and IRF2 function as transcriptional activator and repressor, respectively, of IFNβ and of ISGs. Both factors compete for binding to the same DNA binding element, termed IRF-E, which is found within the promoters of IFNα, IFNβ and many ISGs (Harada *et al.* 1998). Other IRF family members have been shown to play an important role in the regulation of the IFN pathway. For example, p48 (IRF9) was found to be essential for the production of IFNα (Sato *et al.* 2001). Interestingly, the sequence of IRF-E overlaps with ISRE, the site recognized by the transcription factor complex ISGF3, suggesting that these two factors may function redundantly in the regulation of ISGs. Gene targeting studies in mice deficient for p48 and/or IRF1 have shown that p48 and IRF1 regulate distinct genes and are not redundant in the IFN signal transduction pathway, but rather complement one another in both type I and type II IFN responses (Kimura *et al.* 1996). Moreover, IFNα/β can amplify its own expression through a positive feedback loop likely to be dependent on the actions of IRF3 and IRF7 (Marie *et al.* 1998,Sato *et al.* 1998a,Malmgaard 2004). IRF3 is constitutively expressed in the cytoplasm of various cell lines and translocates to the nucleus upon viral infection, where it causes a marked increase in IFNβ expression (Sato *et al.* 1998b). Knockout studies also indicate that IRF7 is involved in the induction of transcription of both IFNα and IFNβ (Sato *et al.* 1998a,Nakaya *et al.* 2001). These observations highlight the complexity of the mechanisms regulating the expression of IFNs and ISGs, and demonstrate the importance of feedback loop mechanisms for the amplification and regulation of IFN signal transduction.

Negative regulation of IFN signalling can be achieved through a variety of mechanisms. IFNs may downregulate the expression of the IFNGR2 mRNA and protein (Pernis *et al.* 1995,Bach *et al.* 1995), although this has not been shown in cells other than T cells. Alternatively, there are at least three families of proteins

known to inhibit the JAK/STAT signalling: protein phosphatases such as SHP-1; Protein Inhibitor of Activated STAT (PIAS) which directly inhibit STAT proteins; and the members of the Suppressors of Cytokine Signalling (SOCS) family (Hilton 1999). SOCS are themselves induced by the JAK-STAT pathway and therefore constitute a classical negative feedback loop that negatively regulates the cellular response to IFNs. Interestingly, the C-terminal SOCS box domain seems to mediate inhibition of IFN signalling by binding to and thereby targeting activated JAKs for proteasomal degradation. Thus, the SOCS box, which is also present in five other distinct protein families, could potentially act as part of an E3 ubiquitin ligase complex that may target a wide range of proteins for ubiquitination and proteasomal degradation (Kile & Alexander 2001,Kile *et al.* 2002).

5.3 ErbB2-Dependent Downregulation of IFN-Inducible Genes

ErbB2 is known to potentiate signalling from other ErbB receptors and it is well established that its overexpression in breast cancer is an indicator of poor patient prognosis (Slamon *et al.* 1987,Ross & Fletcher 1998,Ross & Fletcher 1999). Although it is known that ErbB2 overexpression can translate into signals that lead to enhanced proliferation and survival through the activation of signalling pathways such as the MAPK and PI3K pathways, the mechanistic details and the molecules involved in such responses remain largely unknown. Given the variety of responses resulting from ErbB2 overexpression and the number of proteins reported to be involved in the malignant phenotype observed in ErbB2-positive breast cancers, it is likely that ErbB2 interacts, disrupts and/or cooperates with other signalling pathways to elicit such responses.

In the microarray analysis discussed in Chapter 3, a significant number of ISGs were found to be downregulated in the ErbB2 overexpressing mammary cell line C3.6. Given the role of IFNs in the negative regulation of cellular proliferation, it is possible that this downregulation of IFN signalling may be one mechanism by which ErbB2 confers a more highly proliferative cellular phenotype. There were 48 known IFN-responsive genes represented on the arrays used in this study, and when only these genes were used for SAM analysis, 28 showed significantly different expression levels between the parental and ErbB2 overexpressing cell lines, 16 of which were differentially expressed under serum-starved conditions. These genes are

listed in Table 5.1. Note that although some of the genes were called as significant by SAM, when a t-test was performed independently some of the *p*-values obtained were reasonably high. Nonetheless, there was a clear trend for downregulation of such genes in C3.6 cells compared with HB4a cells, though variability between experiments is an inherent problem of microarray data. Some of the genes were constitutively downregulated in C3.6 cells (represented by a red asterisk in Table 5.1), and therefore these genes are more likely to represent true targets of ErbB2 overexpression rather than of activated signal transduction pathways downstream of ErbB receptors.

Hierarchical clustering of all 48 IFN-related genes showed that such genes can be divided into two groups: genes which are downregulated by ErbB2 and genes whose expression is not affected by ErbB2 overexpression (Figure 5.2). Neither group showed significant changes in gene expression in response to growth factor. Upon closer inspection, the cluster of genes that were significantly downregulated in C3.6 cells were found to be primarily induced by type I IFNs, or to be an important factor in the type I signal transduction pathway. Among the constitutively downregulated genes was p48 (or ISGF3G), the DNA binding component of the ISGF3 transcription complex required for the transcription of a large number of ISGs. Thus, p48 downregulation could be a means by which ErbB2 suppresses the induction of multiple downstream ISGs. This would have the effect of blocking their anti-proliferative action, potentially promoting cell growth. This hypothesis forms the basis on which the following Chapter is based.

While the role of p48 in IFN signalling is reasonably well established, a possible role in oncogenesis is unclear. The importance of p48 in cancer has been analyzed in the context of IFN therapy efficacy. For example, bladder cancer and melanoma cell lines insensitive to the growth inhibitory effects of IFNα were shown to have undetectable or very low levels of p48 expression (Wong *et al.* 1997,Lu *et al.* 2002), whereas IFNγ priming induced the expression of p48 and consequently restored IFN-responsiveness in myeloid leukemia and melanoma cells (Matikainen *et al.* 1997,Wong *et al.* 1998). Conversely, it was shown that p48 expression is directly induced by the protooncogene *c-myc* and that cells lacking p48 are highly susceptible to the cytocidal action of anticancer drugs (Weihua *et al.* 1997b). Indeed, p48 was found to be overexpressed in a subset of uterine and breast tumours, and to confer resistance to anti-microtubule agents in breast cells (Luker *et al.* 2001). Despite such

discrepancies, these findings suggest the involvement of p48 in tumour progression and drug resistance and highlight the need for further research into the role of p48 in cancer development.

Gene ID	Fold Change	t test
G1P2 *	0.17	1.8×10^{-4}
IFITM1 *	0.25	0.01
IFITM1 *	0.29	3.6×10^{-3}
IFITM2 *	0.34	0.01
IFITM2 *	0.36	0.01
OAS1	0.42	0.01
IFIT1 *	0.63	0.04
IRF3	0.65	0.05
PRKR	0.72	0.03
ISGF3G *	0.73	0.01
SP110 *	0.74	0.04
PSME1	0.75	0.03
PSME1	0.76	0.06
ISGF3G *	0.81	0.05
HRMT1L2	0.82	0.08
SP110 *	0.82	0.01

* Genes downregulated at all time points

Table 5.1: Differentially expressed IFN-responsive genes. The table shows differentially expression IFN-responsive genes identified using SAM software when comparing HB4a and C3.6 cells under serum-starved conditions. Fold changes represent the ratio of the average intensity values of gene expression for a given gene in C3.6 cells divided by average intensity value in HB4a cells. Asterisks indicate genes downregulated at all time points when direct SAM comparisons were made between the cell lines at each time point.

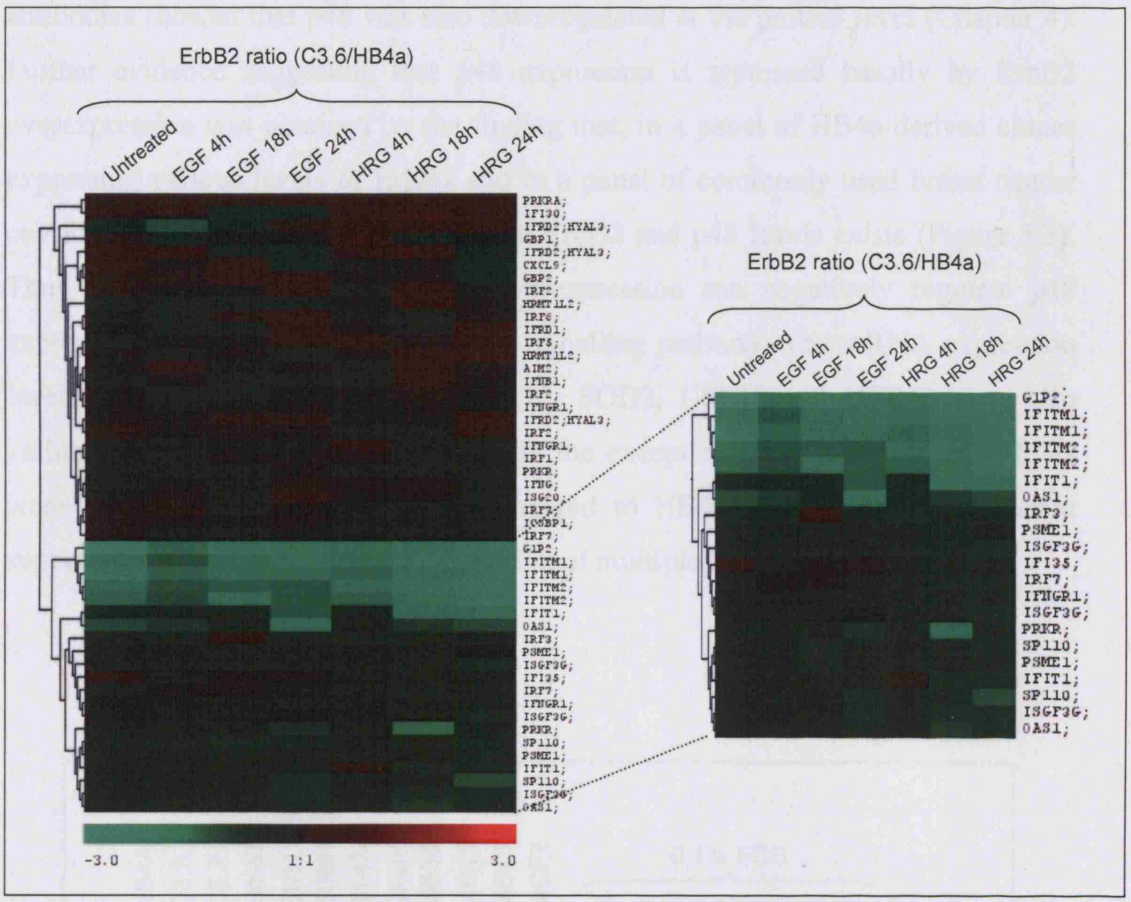

Figure 5.2: Hierarchical clustering of IFN-responsive genes. All 48 IFN-responsive genes represented on the array were clustered together using the ErbB2 ratio to obtain a visual representation of genes differentially expressed in C3.6 cells.

5.4 ErbB2 and p48 Downregulation

The first step taken in order to establish whether indeed ErbB2 negatively regulates p48 expression was to confirm its downregulation in the C3.6 cells. Quantitative real time PCR (qRT-PCR) experiments have confirmed the downregulation of p48 at the mRNA level and immunoblotting using p48-specific antibodies showed that p48 was also downregulated at the protein level (Chapter 4). Further evidence suggesting that p48 expression is repressed basally by ErbB2 overexpression was obtained by the finding that, in a panel of HB4a-derived clones expressing various levels of ErbB2 and in a panel of commonly used breast cancer cell lines, an inverse correlation between ErbB2 and p48 levels exists (Figure 5.3). Thus, it is very likely that ErbB2 overexpression can negatively regulate p48 expression and therefore affect the IFN signalling pathway. The mRNA expression levels of the ISGs OAS1, G1P2, STAT1, SOD2, USP18 and UBP43 were also validated by qRT-PCR (Chapter 4). With the exception of STAT1, all these ISGs were downregulated in C3.6 cells compared to HB4a, linking ErbB2-dependent suppression of p48 with the downregulation of multiple IGSs.

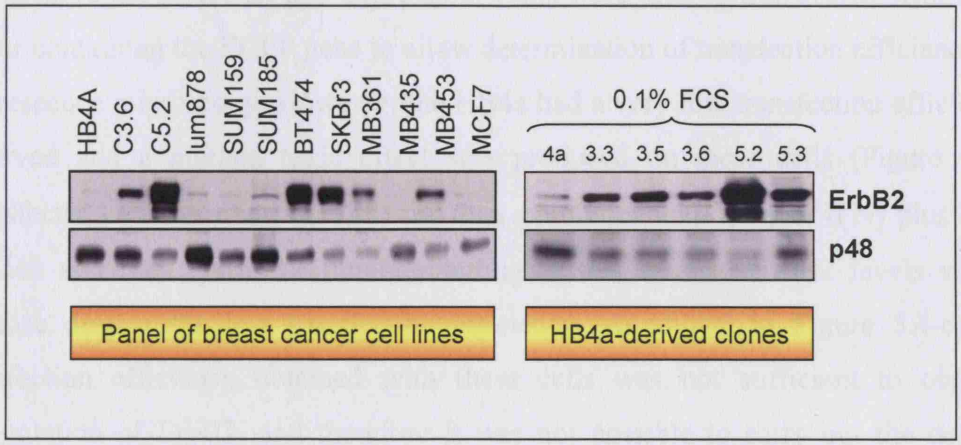

Figure 5.3: Validation of p48 downregulation in C3.6 cells. Lysates from a randomly growing panel of commonly used breast cancer cell lines (left panel) and from serum-starved HB4a-derived clones were immunoblotted using ErbB2 and p48-specific antibodies.

5.4.1 Establishment of the direct repression of p48 by ErbB2: transfection experiments

The best way to determine whether the regulation of p48 by ErbB2 is a true event or just a secondary occurrence caused by unwanted artefacts (such as clonal variation, cellular immortalization effects, cell line-specific gene mutations, etc.) is to observe how p48 is affected when the ErbB2 gene is expressed in cells acutely. Transient transfection techniques offer the most rapid and efficient way to attain this goal. There are several transfection methods that enable the introduction of genes into cells. Due to their simplicity, lipid-based methods are currently the most commonly used, whereby the DNA of interest is combined with cationic lipids to create lipid-DNA complexes that fuse with the cell membrane and are then transported into the cell. However, as with most techniques, there are drawbacks with transfection experiments. Transfection studies are still hampered by the difficulty of transfecting cells with high efficiency. In addition, one must remember that transfections often result in strong expression of individual genes, and the resulting cellular phenotypes may not always represent the true *in vivo* gene function. Nonetheless, it is still a widely used technique that can provide important information on biological function.

Initially, the aim was to transiently transfect HB4a cells with the ErbB2 gene and observe the effect on p48 expression. Cells were also co-transfected with a C2 vector containing the EGFP gene to allow determination of transfection efficiency by fluorescence microscopy. However, the HB4a had a very low transfection efficiency observed and a marked toxic effect was produced on these cells (Figure 5.4). Transfected cells were left for 24h, and then stimulated with IFNγ or IFNγ plus EGF for 24h and then lysed for immunoblotting to test whether ErbB2 levels would increase despite the low transfection efficiency. As shown in Figure 5.4-c, the transfection efficiency obtained with these cells was not sufficient to observe upregulation of ErbB2, and therefore it was not possible to carry out the desired experiments. Transfection protocols were optimized according to manufacturer's instructions, and parameters such as number of starting cells, ratio of transfection reagent to DNA and time of transfection were adjusted with no significant improvement in either efficiency or cell death. Furthermore, various transfection reagents were tested, including Superfect (Qiagen), Metafectene (Biontex), Efectene

(Qiagen) and Fugene6 (Roche). Once again, transfection of HB4a cells was not improved.

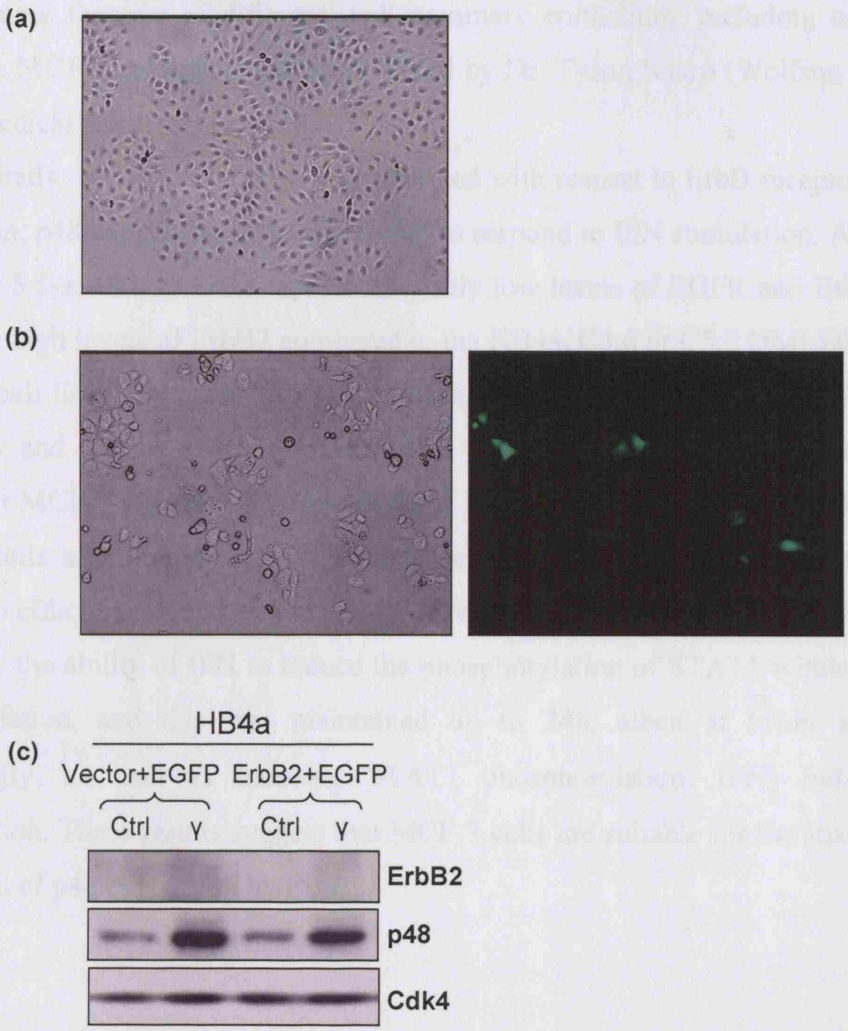

Figure 5.4: Transfection of HB4a cells. (a) Healthy HB4a cells before transfection with vectors containing ErbB2 and EGFP. **(b)** HB4a cells shown 24h after transfection, where a high degree of cell death has occurred (left panel), and low transfection efficiency is observed (right panel). **(c)** Immunoblot experiment of HB4a cells transfected with either an empty vector or with ErbB2 plus EGFP. Cells were either unstimulated or stimulated with IFNγ. Transfection efficiency achieved was not sufficient to upregulate ErbB2 levels. Cdk4 is shown as loading control.

Since efficient transfection of HB4a cells with the ErbB2 gene was not possible, the cell line MCF-7 was employed to carry out similar experiments. These breast adenocarcinoma cells are widely used as a breast cancer model system. They were derived from a pleural effusion, display an epithelial-like morphology and exhibit some features of differentiated mammary epithelium, including oestradiol synthesis. MCF-7 cells were kindly provided by Dr. Tyson Sharp (Wolfson Institute for Biomedical Research, London, UK).

Firstly, MCF-7 cells were characterized with respect to ErbB receptor family expression, p48 expression and their ability to respond to IFN stimulation. As shown in Figure 5.5-a, MCF-7 cells express relatively low levels of EGFR and ErbB2, but relatively high levels of ErbB3 compared to the HB4a, C3.6 or C5.2 HMLECs. None of these cell lines expressed ErbB4. The level of p48 expression in MCF-7 cells is quite low and similar to levels observed in C5.2 cells. This is perhaps surprising given that MCF-7 express very low levels of ErbB2. p48 expression was induced in MCF-7 cells after long-term IFN stimulation (Figure 5.5-b), with EGF treatment having no effect on the expression of p48. IFN signalling is also likely to be intact, as shown by the ability of IFN to induce the phosphorylation of STAT1 within 15mins of stimulation, and this was maintained up to 24h, albeit at lower intensity. Interesingly, as well as inducing STAT1 phosphorylation, IFNγ induced its upregulation. These results suggest that MCF-7 cells are suitable for the study of the regulation of p48 expression by ErbB2.

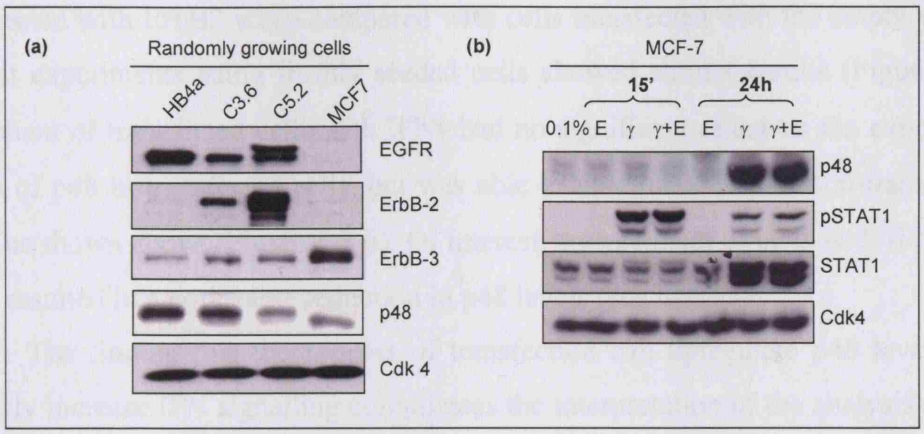

Figure 5.5: Characterization of MCF-7 cells. (a) Randomly growing MCF-7 cells were characterized with respect to ErbB receptor family expression levels, as well as p48 expression levels. **(b)** Consistent with the findings for HB4a and C3.6 cells, p48 was induced following long-term IFN stimulation, and STAT1 phosphorylation was apparent only 15mins after IFN stimulation. Cdk4 was used as a loading control.

Having established MCF-7 cells as an appropriate cellular system, the next step was to transfect them with the ErbB2-containing vector. Fugene6 (Roche) was used for transfection and the protocol was optimized according to the manufacturer by adjusting cell confluency and Fugene6:DNA ratio. The transfection efficiency achieved was above 50%, which was satisfactory for the desired analysis (Figure 5.6-a), although a low level of toxicity was observed, with cells appearing more rounded and a number of detached cells present in the media. Clear expression of ErbB2 was observed after 48h in cells transfected with the ErbB2 vector, but not in cells transfected with the empty vector with more confluent cells yielding a higher level of ErbB2 expression (Figure 5.6-b). Unexpectedly, p48 levels were increased in transfected cells, regardless of whether they were transfected with ErbB2 or empty vector. It is possible that the stress cells undergo during the transfection process could have triggered a response in the IFN pathway and thereby p48 upregulation. This possibility is made more likely by the observation that STAT1 was phosphorylated in the transfected cells, but not in untransfected cells, suggesting that indeed the IFN signalling pathway is activated (Figure 5.6-b). Despite the fact that the IFN signal transduction pathway is activated following transfection and an increase in p48 levels is observed, p48 levels were consistently lower in cells

transfected with ErbB2 when compared with cells transfected with the empty vector. Repeat experiments using freshly seeded cells showed similar results (Figure 5.7). Treatment of transfected cells with IFNγ had no significant effect on the expression levels of p48 in transfected cells, but was able to upregulate p48 in non-transfected cells, as shown above (Figure 5.7-b). Of interest, co-treatment of cells with IFNγ and HRG resulted in a noticeable reduction in p48 levels (see later).

The finding that the process of transfection can upregulate p48 levels and possibly increase IFN signalling complicates the interpretation of the analysis of p48 regulation in response to ErbB2 overexpression. Whilst an ErbB2-inducible system would be highly informative in studying the effects of ErbB2 on p48 expression, due to time constraints these experiments were not possible. It is therefore difficult to obtain a definitive conclusion as to whether ErbB2 overexpression can acutely downregulate p48. Nonetheless, the observed trend of lower p48 levels in ErbB2 transfected cells compared to cells transfected with the empty vector are encouraging findings consistent with the hypothesis that ErbB2 can negatively regulate p48 expression. The remainder of this Chapter will adopt the assumption that indeed this is the case and experiments are presented which try to elucidate the mechanism of p48 downregulation in ErbB2-overexpressing cells.

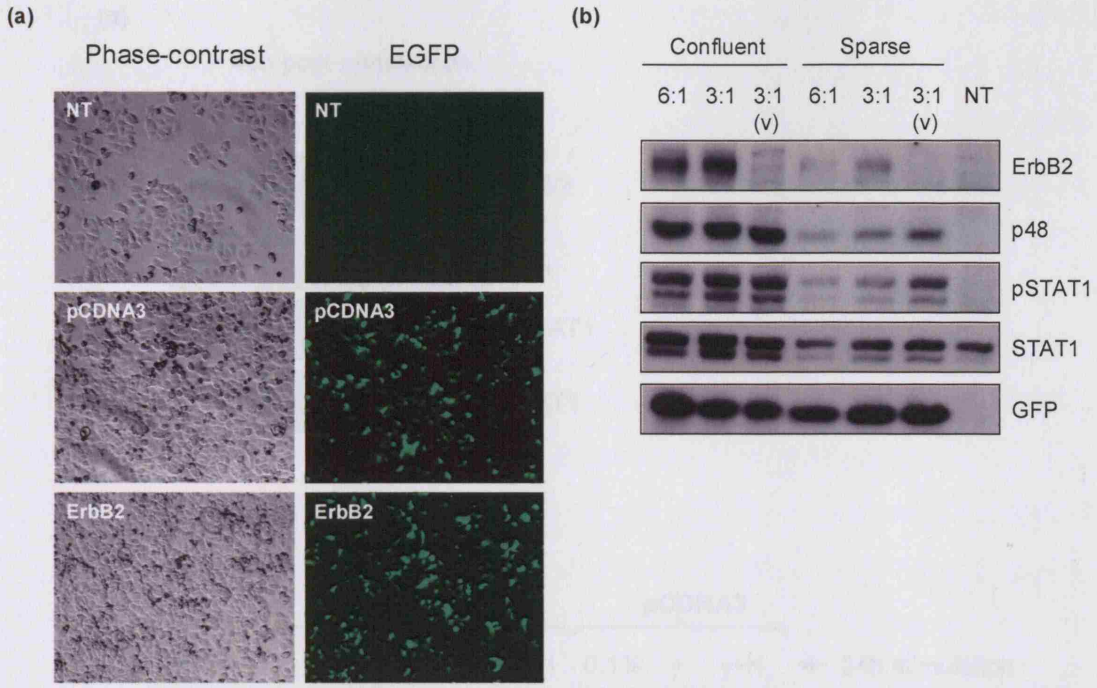

Figure 5.6: MCF-7 cell transfection efficiency. (a) MCF-7 cells shown in phase contrast and by fluorescence microscopy to assess transfection efficiency based on the co-transfection with EGFP. NT: non-transfected; pCDNA3: empty vector. Transfection efficiency was over 50% in all cases. **(b)** Confluent and sparse cell monolayers were transfected with various Fugene6:DNA ratios, here only 6:1 and 3:1 are shown. (v): empty vector; NT: non-transfected.

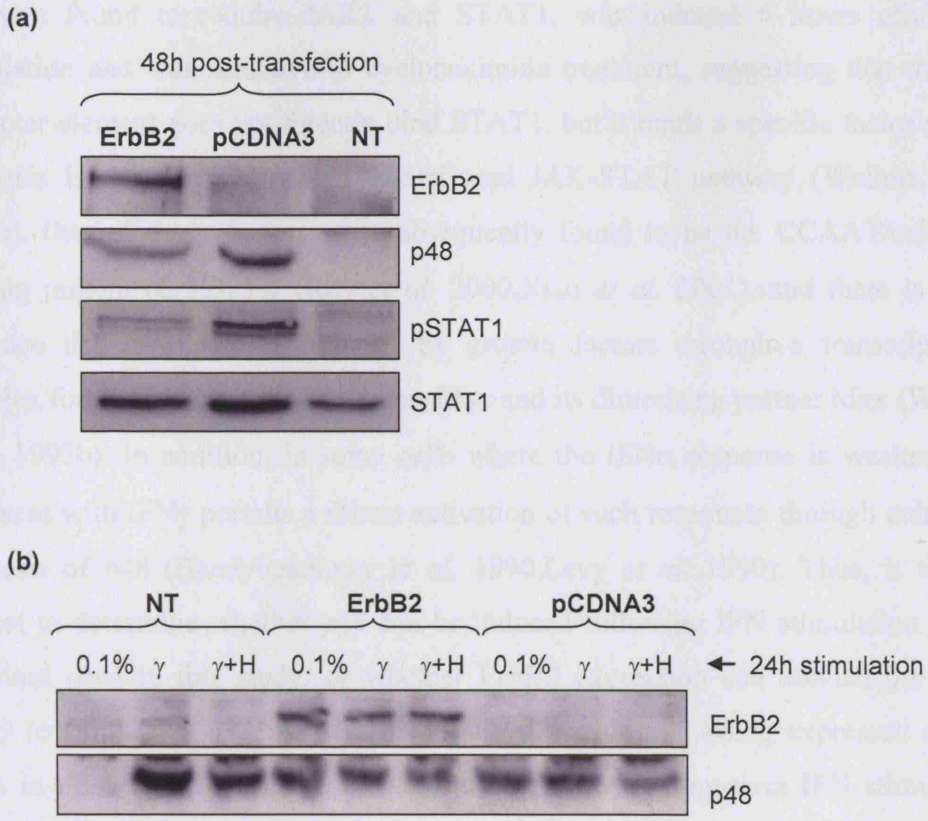

Figure 5.7: ErbB2 transfection of MCF-7 cells and p48 expression. **(a)** Freshly seeded MCF-7 cells were transfected with either ErbB2 or empty vector and expression levels of p48 were assessed. Phosphorylation levels of STAT1 were also investigated. **(b)** Experimental repeat where transfected and untransfected cells were further treated with IFNγ or IFNγ in combination with HRG for 24h. As seen in (a), p48 levels are higher in transfected cells prior to IFN treatment. In addition, IFN treatment has no effect on p48 levels in the transfected cells. NT: non-transfected

5.5 The Effect of Growth Factor Stimulation on IFN Signalling

The experiments above suggest that p48 is downregulated in cells expressing high levels of ErbB2. However, the mechanisms by which ErbB2 affects the expression level of p48 are not clear. There are several possible ways in which ErbB2 could affect p48 expression, for example by blocking p48 transcription or by increasing the rate of p48 protein degradation. Very little is known about how p48 levels are regulated in the cell. There is evidence that p48 expression is induced by IFNγ through a novel regulatory element in the p48 promoter termed GATE (gamma activated transcriptional element) (Weihua *et al.* 1997a). Transcriptional induction of

p48 was found to require JAK1 and STAT1, was induced 6 hours after IFN stimulation and was sensitive to cycloheximide treatment, suggesting that the p48 promoter element does not directly bind STAT1, but it binds a specific factor whose synthesis is dependent on the IFN-activated JAK-STAT pathway (Weihua *et al.* 1997a). One of these factors was subsequently found to be the CCAAT/enhancer binding protein (C/EBP)-β (Roy *et al.* 2000,Xiao *et al.* 2001), and there is some evidence that p48 can be induced by growth factors through a transcriptional complex formed by the proto-oncogene Myc and its dimerizing partner Max (Weihua *et al.* 1997b). In addition, in some cells where the IFNα response is weaker, pre-treatment with IFNγ permits a robust activation of such responses through enhanced synthesis of p48 (Bandyopadhyay *et al.* 1990,Levy *et al.* 1990). Thus, it was of interest to determine whether p48 can be induced following IFN stimulation in the cell lines used in this study, or whether ErbB2 expression can abolish the cell's ability to synthesize p48. As seen in Figure 5.8-a, despite being expressed at low levels in C3.6 cells, p48 can still be induced following long-term IFN stimulation (24h and 48h). Although HB4a cells have relatively high levels of p48 in the un-stimulated state, p48 is induced even further following 24h IFN stimulation. These results are consistent with the above reported observation, where p48 induction is delayed after IFN stimulation because of the requirement of a newly synthesized factor to induce its synthesis. Interestingly, p48 synthesis occurred in response to both type I and type II IFNs. Once again, growth factor stimulation had little effect on p48 protein expression. Further confirmation that IFN signalling is functional in C3.6 cells despite ErbB2 overexpression, cells were stimulated with EGF and HRG as well as type I and II IFNs for 4h and immunoblotted for the ISGs MxA, MxB and MnSOD (Figure 5.8-b). Mx proteins are induced only by type I IFNs, whereas MnSOD expression can be induced by both type I and type II IFNs. C3.6 cells were able to retain their ability to induce these ISGs, and the specificity of IFN signalling was also maintained, as the Mx proteins were only induced by IFNβ treatment (Figure 5.8-b).

To further test whether the IFN pathway is functional in these cells, proliferation assays were performed in randomly growing cells treated with increasing concentrations of both IFNβ and IFNγ (Figure 5.9). Treatment with both types of IFNs resulted in decreased cellular proliferation of both the HB4a and C3.6

cell lines in a dose-dependent manner. Similarly, both types of IFNs caused an inhibition of proliferation in C5.2 cells, a clone derived from HB4a cells expressing even higher levels of ErbB2 (Figure 5.9) (Harris *et al.* 1999). IFNγ was more potent at inhibiting cellular proliferation, as it was able to induce almost maximal inhibition at lower concentrations compared to IFNβ, particularly in C3.6 cells. At high concentrations, IFNγ was able to induce a higher degree of inhibition of proliferation in the ErbB2 overexpressing cells (C3.6 and C5.2), although the relevance of these findings is still unknown. The above results demonstrate that ErbB2 overexpression does not impair the IFN response and that the IFN signalling machinery is intact in these cells.

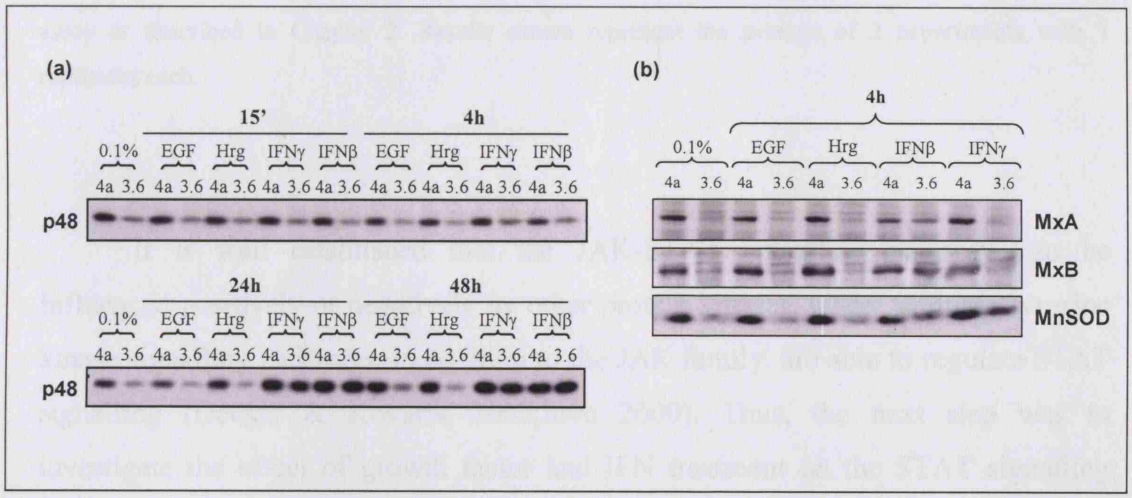

Figure 5.8: IFN treatment can induce p48 upregulation in both normal and ErbB2-overexpressing cell lines. (a) HB4a and C3.6 cells were serum-starved for 48h and stimulated with EGF, HRG, IFNγ or IFNβ for the time points shown and lysates immunoblotted with p48-specific antibodies. **(b)** HB4a and C3.6 cells were serum-starved for 48h and stimulated for 4h with EGF, HRG, IFNγ or IFNβ. Samples were immunoblotted using antibodies specific for the ISGs MxA, MxB and MnSOD.

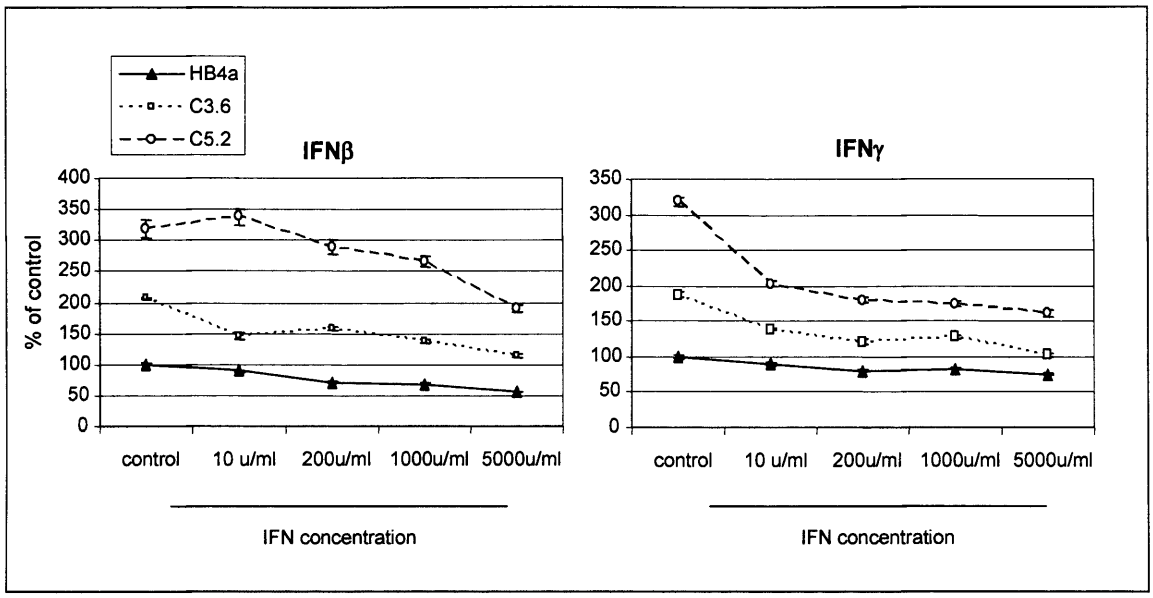

Figure 5.9: IFN dose proliferation assays. Randomly growing (in 10% serum) HB4a, C3.6 and C5.2 (an HB4a-derived clone which contains even higher levels of ErbB2) cells were treated with increasing concentrations of IFNβ or IFNγ for 48h. Proliferation rate was measure using the MTT assay as described in Chapter 2. Results shown represent the average of 2 experiments with 7 replicates each.

It is well established that the JAK-STAT signalling pathway can be influenced positively or negatively by other protein kinases, where multiple tyrosine kinase signalling pathways, in addition to the JAK family, are able to regulate STAT signalling (Decker & Kovarik 2000,Jove 2000). Thus, the next step was to investigate the effect of growth factor and IFN treatment on the STAT signalling pathway to determine whether aberrant STAT signalling could be a mechanism of interferon signalling disruption in the C3.6 cells (Figure 5.10-a). As previously reported (David *et al.* 1996), EGF stimulation was able to induce the phosphorylation of STAT1. This was much weaker in C3.6 cells, perhaps reflecting the lower level of EGFR in these cells which would result in lower signalling potency through this receptor and may be one explanation for the lower levels of p48 expression in the C3.6 cells. STAT1 and STAT3 phosphorylation was equal in both cell lines following IFN stimulation, (Figure 5.10-b). IFNγ induced a longer lived STAT1 activation than IFNβ, with phosphorylation levels still at maximal intensity at the 4h time point. STAT1 phosphorylation by IFNγ was also apparent at 24h and was

equivalent in the two cell lines (data not shown). The effect of IFN stimulation on the ErbB receptor family and its major downstream signalling components was also analysed. Growth factor-specific receptor downregulation occurred, and this is part of the normal negative regulatory mechanisms of ErbB signalling (Timms *et al.* 2002). However, IFN stimulation had no effect on the expression level of any of the ErbB receptors, even at prolonged stimulation times (Figure 5.10-c). MAPK and PI3K activation were observed following EGF and HRG treatment as expected (Figure 5.11). Akt phosphorylation was slightly higher in HRG stimulated cells, particularly C3.6 cells, reflecting the potency of HRG-mediated PI3K activation through ErbB2/ErbB3 heterodimers. On the other hand, MAPK activation was longer lived in EGF-treated cells compared to HRG. Both types of IFN induced a short-lived activation of the MAPK pathway, which returned to basal levels of phosphorylation by 4h. The expression of two CDK inhibitors was also examined to determine whether IFN or growth factors could affect key cell cycle regulators. The expression of p21 was induced by both growth factors after 4h stimulation, and it was induced more strongly in C3.6 cells whilst levels of p27 were lower in C3.6 cells, confirming previous findings reported in our lab (Timms *et al.* 2002). IFN stimulation had little effect on the expression of these CDK inhibitors, although longer time points may be required to see the reported induction which is required for IFNs to inhibit proliferation (Figure 5.11).

These results show that the JAK-STAT signalling pathway can be induced by ErbB-specific ligands, and indeed signalling pathways downstream of ErbB receptors can also be induced following IFN stimulation. Thus, the IFN pathway does not function autonomously, confirming previous literature reports and showing that these cell lines reflect the complexity of this signalling pathway faithfully.

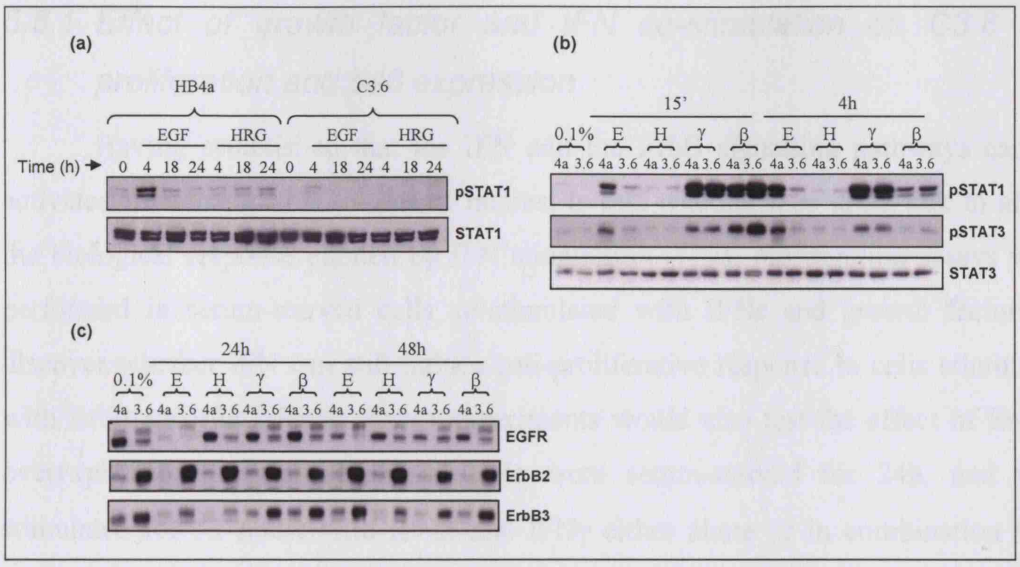

Figure 5.10: Effect of growth factor and IFN treatment on STAT signalling and ErbB family members. Antibodies specific for the phosphorylated form of STAT1 and STAT3 were used to test the activation of the STAT signalling pathway upon growth factor and IFN stimulation. **(a)** STAT1 is phosphorylated by EGF treatment, and this effect is more potent in HB4a cells, which express higher levels of EGFR. **(b)** Both STAT1 and STAT3 become phosphorylated following EGF, IFNβ and IFNγ stimulation. **(c)** Expression of ErbB family members is not affected by IFN stimulation.

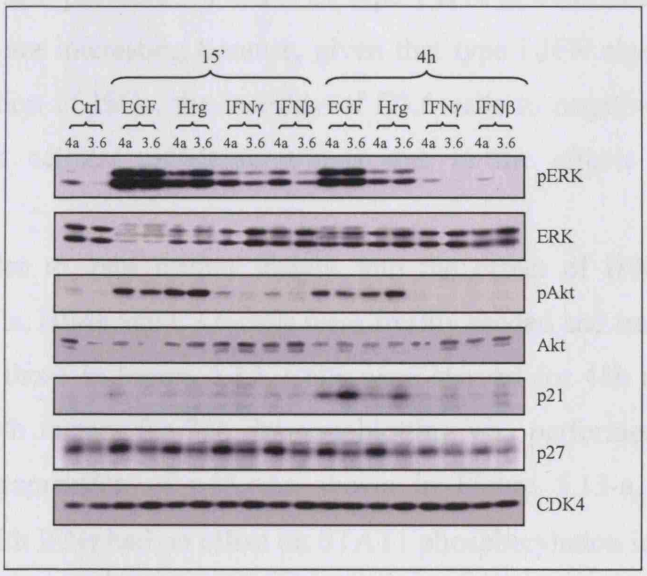

Figure 5.11: Effect of growth factor and IFN treatment on MAPK and PI3K signalling. HB4a and C3.6 cells were serum-starved for 48h and stimulated with EGF, HRG, IFNβ or IFNγ for 15 minutes or 4 hours. Antibodies specific for the phosphorylated forms of ERK and Akt were used to test for activation of MAPK and PI3K pathways, respectively. The induction of the CDK inhibitors p21 and p27 was also tested. Cdk4 was used as the loading control.

5.5.1 *Effect of growth factor and IFN co-stimulation on C3.6 cell proliferation and p48 expression*

Having established that the IFN and the ErbB signalling pathways can be activated interchangeably, it was of interest to test whether they cross-talk to affect the biological response elicited by IFN stimulation. Thus, proliferation assays were performed in serum-starved cells co-stimulated with IFNs and growth factors to discover whether IFN can still induce anti-proliferative response in cells stimulated with ErbB-specific ligands. These experiments would also test the effect of ErbB2 overexpression on this response. Cells were serum-starved for 24h, and then stimulated for 72 hours with IFNβ and IFNγ either alone or in combination with growth factors. As seen in Figure 5.12, stimulation with EGF or HRG alone caused a marked increase in cellular proliferation in both HB4a and C3.6. IFN treatment had no effect on the proliferation of serum-starved cells, indicating that cells were not undergoing apoptosis. When cells were co-stimulated with IFN and growth factor, IFNγ was able to significantly inhibit growth factor-induced proliferation of both cell lines (Figure 5.12, right panel), but IFNβ was only able to produce the same effect in HB4a cells (left panel). This suggests that growth factor treatment somehow overrides the anti-proliferative effect of type I IFN in cells overexpressing ErbB2. These results are interesting because, given that type I IFN signalling requires p48 for the induction of ISGs, the inability of C3.6 cells to negatively regulate growth factor-induced cellular proliferation may due to the effects of ErbB2 on p48 expression.

In order to gain further insight into the effect of IFN on growth factor stimulated cells, HB4a and C3.6 cells were freshly seeded and treated under identical conditions to those in Figure 5.12. Cells were starved for 48h and stimulated with IFN +/- growth factors for 72h. Immunoblotting was performed on cell lysates to monitor the expression of p48. As shown in Figure 5.13-a, growth factor co-stimulation with IFNγ had no effect on STAT1 phosphorylation in the HB4a cells but was reduced compared to IFNγ alone in the C3.6 cells. IFNβ-induced STAT1 phosphorylation was no longer detected at this later time point. There was a very weak STAT3 phosphorylation retained in growth factor-treated HB4a cells. Importantly, induction of p48 expression was observed, as expected, by both IFNβ and IFNγ, however, when cells were co-treated with growth factors, the induction of

p48 by IFNγ was abrogated in the C3.6 cells and reduced in these cells by IFNβ co-treatment (Figure 5.13-a). These experiments were repeated on freshly seeded cells treated under the same conditions using two different p48-specific antibodies, and similar results were obtained (Figure 5.13-b). Quantitative real time PCR experiments showed that the IFNγ-mediated induction of p48 expression occurs at the transcriptional level (Figure 5.13-c). However, EGF co-stimulation was not able to inhibit the induction of p48 by IFNγ as seen in the western blot data, suggesting that this effect is a post-transcriptional event. Thus, growth factor stimulation inhibits IFNγ-mediated induction of p48 expression in ErbB2-overexpressing cells. Importantly, this was independent of changes in STAT1 phosphorylation.

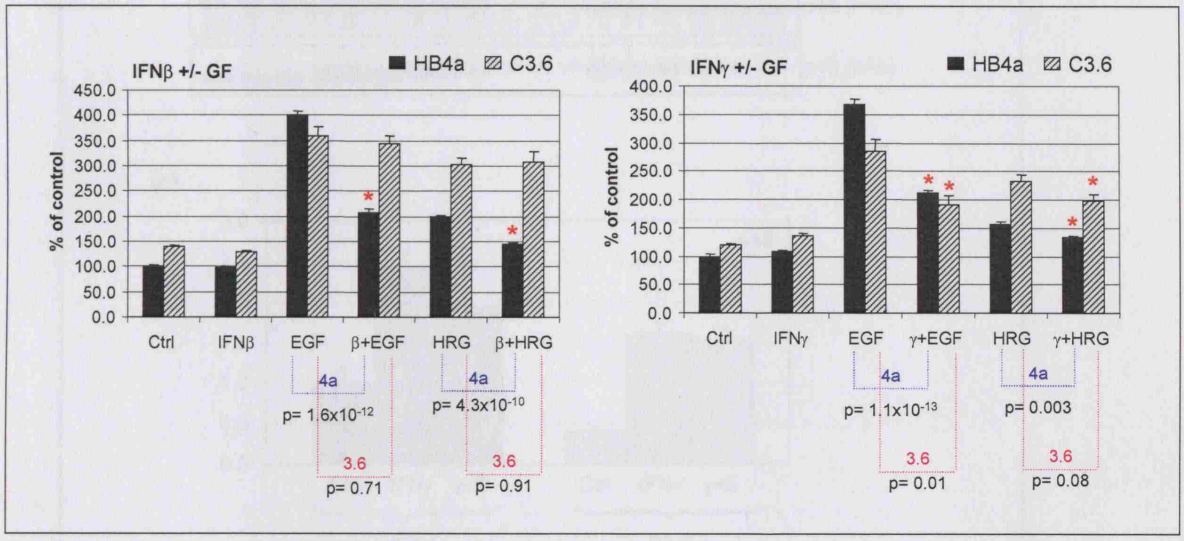

Figure 5.12: IFN-mediated inhibition of growth factor-induced cell proliferation. Proliferation assays were performed on serum-starved cells or cells stimulated for 72h with IFNβ or IFNγ +/- growth factors. Results represent the average of three experiments, analysing 8 replicates each. Red asterisks represent statistically significant inhibition of proliferation in IFN+GF stimulated cells compared to cells stimulated only with GF (*p*-values are shown below).

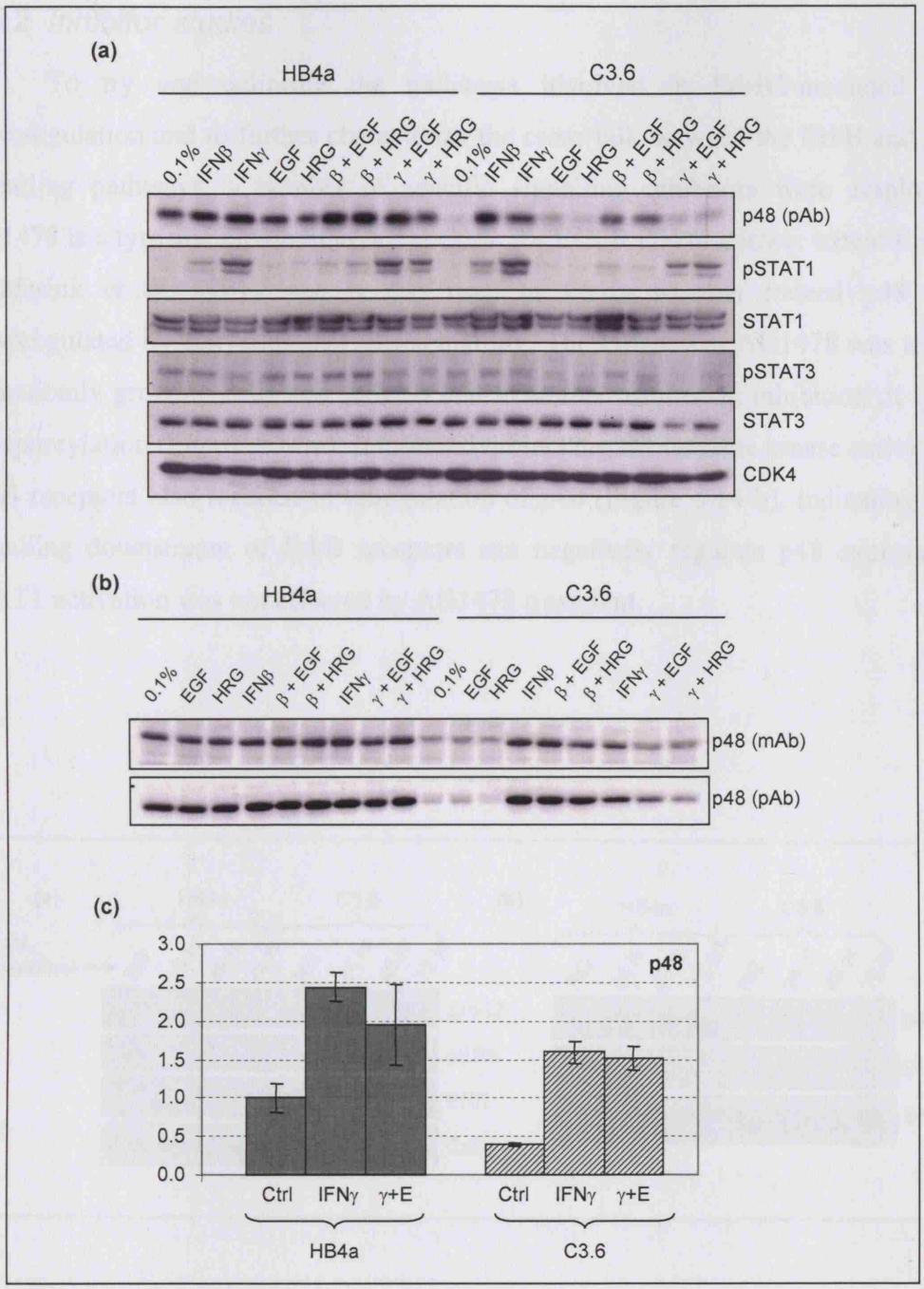

Figure 5.13: Effect of IFN/growth factor co-stimulation on p48 expression and STAT signaling pathway. (a) Cells were serum-starved for 48h and stimulated for 72h with growth factor and IFN alone or in combination. No significant differences in STAT signaling were observed between the two cell lines, but the IFNγ-induced expression of p48 was abrogated by growth factors in C3.6 cells. **(b)** Freshly plated cells were treated as in (a) and p48 expression was evaluated using two different p48 specific antibodies (mAb: Signal Transduction Laboratories; pAb: Santa Cruz). **(c)** Freshly seeded HB4a and C3.6 cells were serum-starved for 48h and stimulated with IFNγ or IFNγ in combination with EGF for 48h. Total RNA was isolated, reverse transcribed and quantitative real time PCR was performed as described in Chapter 4 to measure changes in mRNA expression of p48.

5.5.2 Inhibitor studies

To try and delineate the pathways involved in ErbB2-mediated p48 downregulation and to further characterize the cross-talk between the ErbB and IFN signalling pathways, a number of specific signalling inhibitors were employed. AG1478 is a tyrosine kinase inhibitor specific for EGFR and to a lower extent ErbB2 (Lenferink *et al.* 2001), and it was used to verify whether indeed p48 was downregulated by activation of ErbB signalling. The efficacy of AG1478 was tested in randomly growing cells and showed that treatment resulted in inhibition of ERK phosphorylation (Figure 5.14-a). Importantly, blocking the tyrosine kinase activity of ErbB receptors also resulted in upregulation of p48 (Figure 5.14-b), indicating that signalling downstream of ErbB receptors can negatively regulate p48 expression. STAT1 activation was not affected by AG1478 treatment.

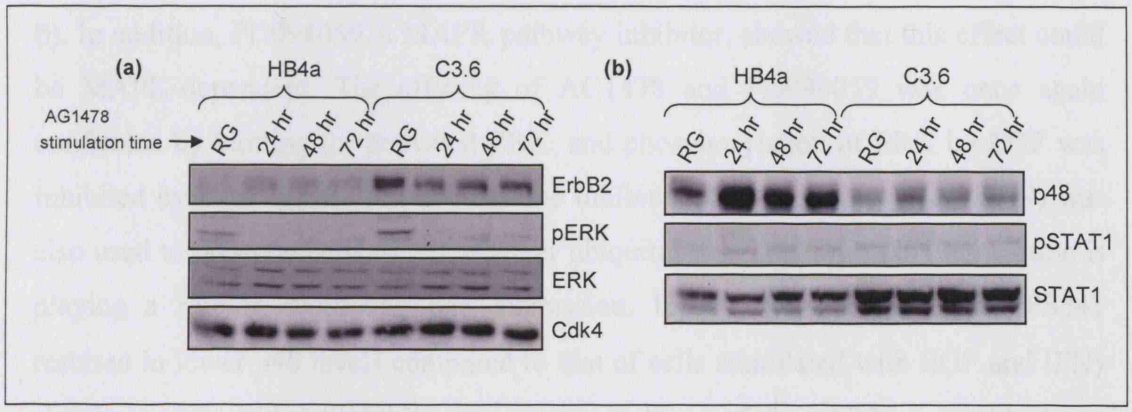

Figure 5.14: AG1478 time course and efficacy testing. (a) Randomly growing (RG) cells were treated with the ErbB inhibitor AG1478 for 24, 48 or 72 hours and immunoblotting was performed using antibodies specific for the phosphorylated form of ERK and total ERK to test for the efficacy of AG1478 inhibition. **(b)** The same cellular lysates were used to test for p48 expression and STAT1 activation in response to ErbB inhibition.

In addition to establishing that the negative regulation of p48 is mediated by ErbB receptors, it was necessary to determine whether the growth factor-mediated inhibition of p48 upregulation by IFNs is also mediated by ErbB receptors. Thus, HB4a and C3.6 cells were serum-starved and pre-treated with AG1478 for 1h and then stimulated with IFNγ alone or in combination with EGF. In C3.6 cells, p48 expression was first detected after 8h IFNγ stimulation and was maximally induced by 72h (Figure 5.15-a). As previously shown, EGF treatment attenuated IFNγ-induced p48 expression at 72h but this effect was not observed in AG1478 treated cells, demonstrating that blocking ErbB signalling can restore the ability of IFNγ to upregulate p48 expression. These results demonstrate that the failure of IFNγ to induce p48 expression is caused by the activation of ErbB signalling, which is more marked in C3.6 cells, suggesting that ErbB2 overexpression enhances this effect. Cell were also pre-treated with the translation inhibitor cycloheximide (CHX), which inhibited the induction of p48 in C3.6 cells, confirming previously published results that p48 expression is dependent on new protein synthesis (Weihua *et al.* 1997a). Repeat experiments using freshly seeded cells confirmed these findings (Figure 5.15-b). In addition, PD098059, a MAPK pathway inhibitor, showed that this effect could be MAPK-dependent. The efficacy of AG1478 and PD098059 was once again confirmed by blotting for activated ERK, and phosphorylation of ERK by EGF was inhibited by both agents. The proteasome inhibitor PS341 (Adams *et al.* 1999) was also used to pre-treat cells to test whether ubiquitin-dependent protein degradation is playing a role in regulating p48 expression. Indeed, pre-treatment with PS341 resulted in lower p48 levels compared to that of cells stimulated with EGF and IFNγ simultaneously without inhibitor pre-treatment. None of the inhibitors used had any effect on STAT1 phosphorylation by IFNγ.

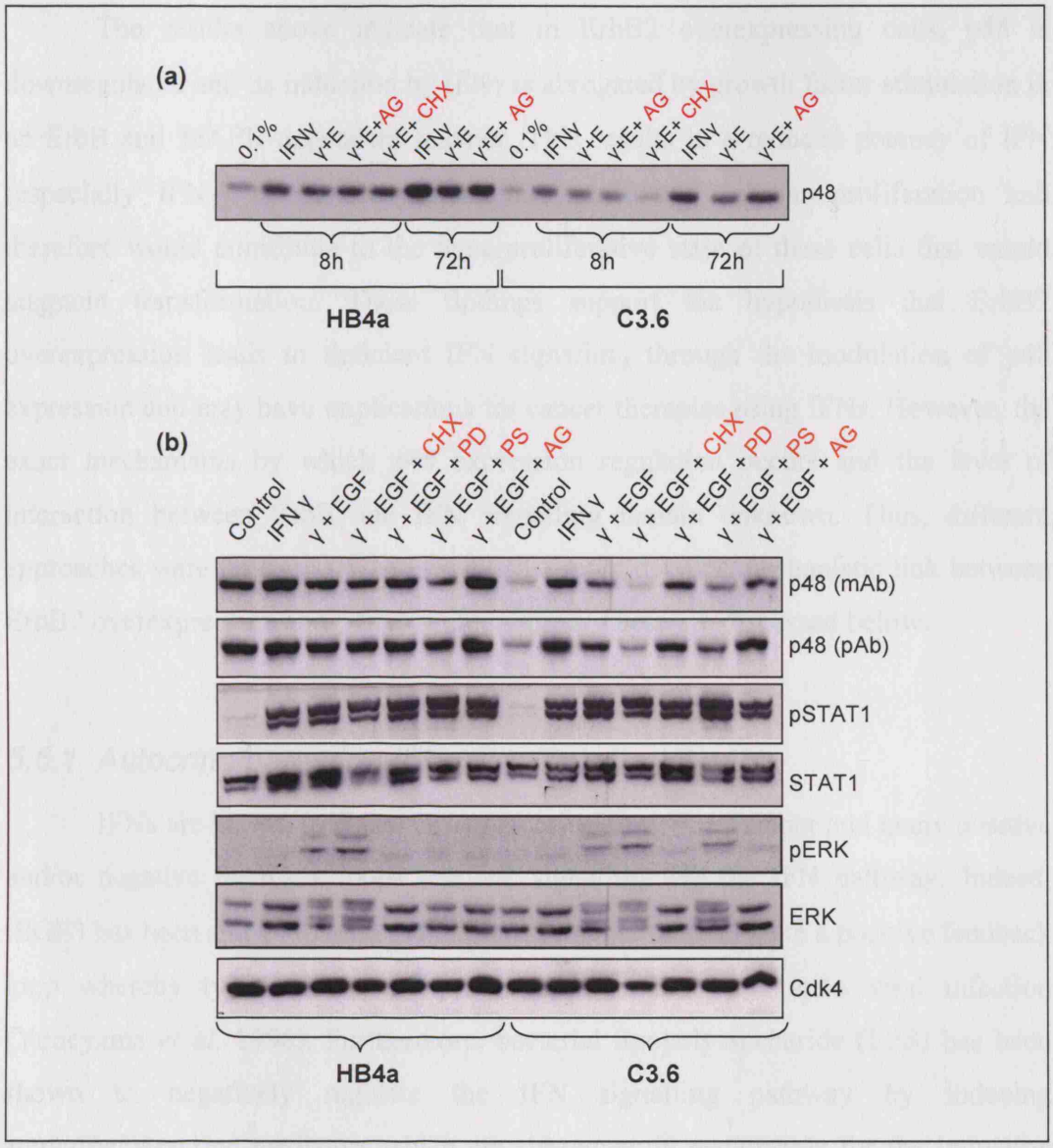

Figure 5.15: Inhibitor studies and the effect of IFN/growth factor co-stimulation on p48 expression. (a) HB4a and C3.6 cells were serum-starved for 24h and pre-treated for 1h with the ErbB kinase inhibitor AG1478 or with cyclohexamide (CHX). Cells were then stimulated with IFNγ alone or in combination with EGF for 8h and 72h. Suppression of IFNγ-induced p48 expression by EGF was restored by AG pre-treatment. **(b)** Repeat and extension of inhibitor studies in cells pre-treated with AG1478, CHX as well as PD098059 (MAPK inhibitor) and PS341 (proteasome inhibitor). Cells were pre-treated with inhibitors for 1h and stimulation with IFN□ alone or with EGF for 48h.

5.6 Elucidation of the Mechanisms of ErbB2-Dependent Downregulation of p48 and Cross-talk with IFN Signalling

The results above indicate that in ErbB2 overexpressing cells, p48 is downregulated and its induction by IFNγ is abrogated by growth factor stimulation in an ErbB and MAPK-dependent manner. This results in a reduced potency of IFN (especially IFNγ) to inhibit growth factor-mediated cellular proliferation and therefore would contribute to the hyperproliferative state of these cells that would augment transformation. These findings support the hypothesis that ErbB2 overexpression leads to deficient IFN signalling through the modulation of p48 expression and may have implications for cancer therapies using IFNs. However, the exact mechanisms by which p48 expression regulation occurs and the level of interaction between ErbB and IFN signalling remain unknown. Thus, different approaches were taken to try and establish a more detailes mechanistic link between ErbB2 overexpression and p48 downregulation. These are discussed below.

5.6.1 Autocrine/paracrine IFN signalling

IFNs are known to signal in an autocrine/paracrine manner and many positive and/or negative feedback loops regulate signalling via the IFN pathway. Indeed, ISGF3 has been shown to bind to the IFNβ promoter and mediate a positive feedback loop whereby type I IFNs are produced at higher rates upon viral infection (Yoneyama *et al.* 1996). Furthermore, bacterial lipopolysaccharide (LPS) has been shown to negatively regulate the IFN signalling pathway by inducing autocrine/paracrine mediators which are subsequently responsible for the induction of SOCS-1 (Crespo *et al.* 2000). Activation of STAT family members through autocrine/paracrine mechanisms has also been reported. In a panel of six breast carcinoma cell lines, a serum-dependent autocrine/paracrine activation of STAT3 was found to correlate with cell proliferation (Li & Shaw 2002). Thus, it was of interest to determine whether HB4a and C3.6 cells are able to modulate the IFN response through autocrine/paracrine signalling and whether ErbB2 overexpression can affect such signalling.

In order to test any differences in autocrine/paracrine signalling in these cells, media swap experiments were carried out as described in Materials & Methods. As seen in Figure 5.16-a, conditioned media taken from serum-stimulated HB4a cells

had no effect on p48 expression or STAT1 and STAT3 phosphorylation in C3.6 cells, and *vice versa*. Similar results were obtained with conditioned media taken from cells stimulated with growth factors (data not shown). When conditioned media was taken from cells stimulated with IFNs, no effect on p48 expression was seen in either cell line stimulated with such conditioned media for 15 minutes. However, there was a clear increase in STAT1 and STAT3 phosphorylation with IFNγ-conditioned media (Figure 5.16-b), suggesting that indeed the JAK-STAT signalling pathway can be activated through autocrine/paracrine means. However, no differences were seen between the two cell lines, and therefore autocrine/paracrine signalling is unlikely to be a mechanism by which ErbB2 overexpression deregulates IFN signalling in C3.6 cells. The short stimulation time examined is probably the reason why p48 levels remain unaffected by IFNγ-conditioned media.

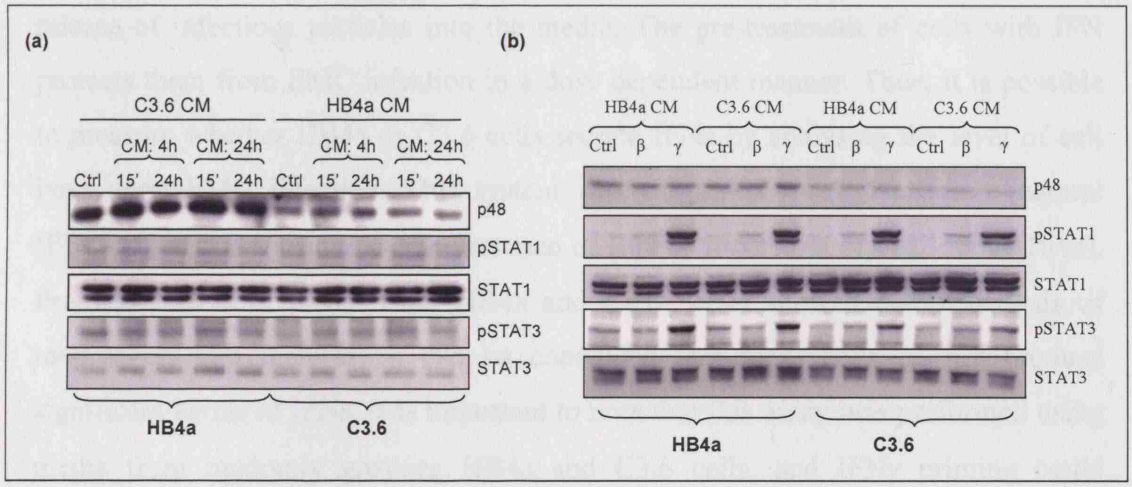

Figure 5.16: Autocrine/paracrine signalling and the IFN pathway. (a) HB4a cells were treated with conditioned media (CM) from serum-treated C3.6 cells and *vice versa* for 15 minutes or 24 hours. No increase in p48 expression or STAT activation were observed. **(b)** HB4a and C3.6 cells were treated for 15 minutes with conditioned media from both HB4a and C3.6 cells stimulated with either IFNβ or IFNγ. While p48 protein levels remained unaffected (which could be because of the short stimulation time), IFNγ treatment induced strong autocrine/paracrine signalling and was able to activate STAT1 and STAT3 in both cell lines equally.

It has been reported that IFNγ priming, i.e. pre-treatment of cells with IFNγ prior to stimulation with type I IFN, can induce a positive feedback loop which results in a 10-fold increased activation of ISGF3 compared to un-primed cells (Levy *et al.* 1990). This probably occurs due to increased production of type I IFNs through IFN-induced IFN production that results in a positive feedback loop leading to increased autocrine signalling (Marie *et al.* 1998,Sato *et al.* 1998a). Such an autocrine loop could therefore account for the activation of STATs observed in cells stimulated with IFNγ-conditioned media. An alternative hypothesis is that the activation of STATs occurs as a consequence of the production of other secreted agents, such as growth factors or other cytokines that are not related to the IFN signalling pathway. To test for IFN autocrine factors, viral protection assays were performed in collaboration with Prof. Ian Kerr (Cancer Research UK). In this assay, the human lung epithelial cells A549 were pre-treated with culture media derived from HB4 or C3.6 cells and then were infected with encephalomyocarditis (EMC) virus. EMC infection of cell cultures results in the lysis of the infected cell and release of infectious particles into the media. The pre-treatment of cells with IFN protects them from EMC infection in a dose dependent manner. Thus, it is possible to measure whether HB4a or C3.6 cells secrete IFNs by analysing the level of cell lysis upon EMC infection. This system was calibrated using Wellferon (natural IFNα-n1) and is sensitive to the presence of IFN at concentrations as low as 1u/ml. Pre-treatment with media from HB4a and C3.6 cells showed complete lysis of monolayers and therefore it can be concluded that these cells do not produce significant levels of IFNs. It is important to note that this assay was performed using media from randomly growing HB4a and C3.6 cells, and IFNγ priming could nonetheless induce secretion of IFNs through the positive feedback loop. Thus, further tests are needed to establish which agent is responsible for STAT1 activation by conditioned media. However, a trivial explanation could be that although cells were washed thoroughly before addition of fresh media for conditioning, there is the possibility that some IFNγ is carried over, and may explain the results.

5.6.2 Cellular localization studies

The subcellular localization of proteins is used as an important regulatory mechanism of protein activity within cells. A classic example of this is provided by the 14-3-3 family of proteins. These proteins appear to play an important role in the regulation of signal transduction, apoptosis and cell cycle checkpoint control by altering the subcellular location of their various binding partners, which include the pro-apoptotic protein BAD and the cell cycle regulator Cdc25 (Muslin & Xing 2000). Thus, in order to better understand the differential protein expression of p48 between the HB4a and C3.6 cells and whether this can have an effect on cellular localization, the distribution of p48 was assessed by immunofluorescence microscopy. The cellular localization of STAT1 was also investigated. Cells were grown to sub-confluency on coverslips, serum-starved for 24 hours and treated for 15 minutes or 24 hours with both IFNβ and IFNγ alone or in the presence of EGF. As seen from Figure 5.17, p48 expression is strictly nuclear in HB4a cells, and is also present at very low levels in the nuclei of C3.6 cells. STAT1 staining was seen primarily in the cytoplasm, but some nuclear staining was also observed in both cell lines. By contrast, STAT2 was exclusively located to the cytoplasm of both cell lines. The staining pattern for p48, STAT1 and STAT2 is identical to that previously reported in human fibroblasts (Koster & Hauser 1999). The cytoplasmic and nuclear staining observed for STAT1 may be a reflection of its ability to shuttle between the cytoplasm and the nucleus of normal, unstimulated cells in a phosphorylation-independent manner (Meyer *et al.* 2002). The cellular localization of p48 was unaffected by treatment with IFNβ or IFNγ for 15 minutes or 24 hours (Figure 5.18-a). In C3.6 cells, p48 levels were increased in the nucleus after 24 hour IFN treatment, in agreement with the western blotting data presented earlier. STAT1, on the other hand, rapidly translocates into the nucleus following IFN stimulation, particularly IFNγ (Figure 5.18-b). STAT1 remained nuclear for a longer period of time in response to IFNγ, whereas after 24h IFNβ stimulation STAT1 was mainly cytoplasmic reflecting its deactivation. Once again, such prolonged activation of STAT1 by IFNγ compared to IFNβ is in agreement with western blotting data presented previously (Figure 5.10) This effect was similar for both cell lines, and therefore it is not likely that ErbB2 affects IFN signalling by altering the cellular localization of components of the STAT signalling pathway.

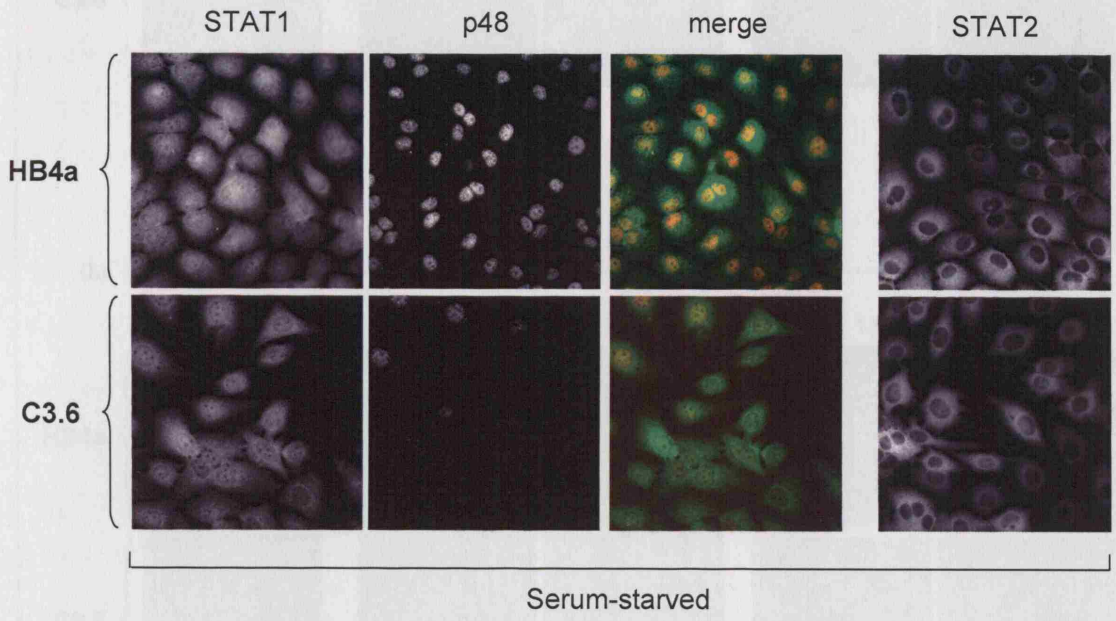

Figure 5.17: Cellular localization studies for p48, STAT1 and STAT2. HB4a and C3.6 cells were grown to sub-confluency on glass coverslips and serum-starved for 24h. Cells were then fixed in 4% paraformaldehyde, permeabilized using 0.2% Triton and double-stained with rabbit antibodies specific for STAT1 or STAT2 and mouse antibodies specific for p48. Secondary antibodies used were anti-rabbit FITC-conjugated and anti-mouse TRTC conjugated. Coverslips were mounted onto microscope slides and allowed to dry. Protein staining was visualized using a x40 magnification on a cooled CCD camera fluorescence microscope. Non-specific staining was tested by adding secondary antibody alone. No staining was observed in these negative controls (data not shown).

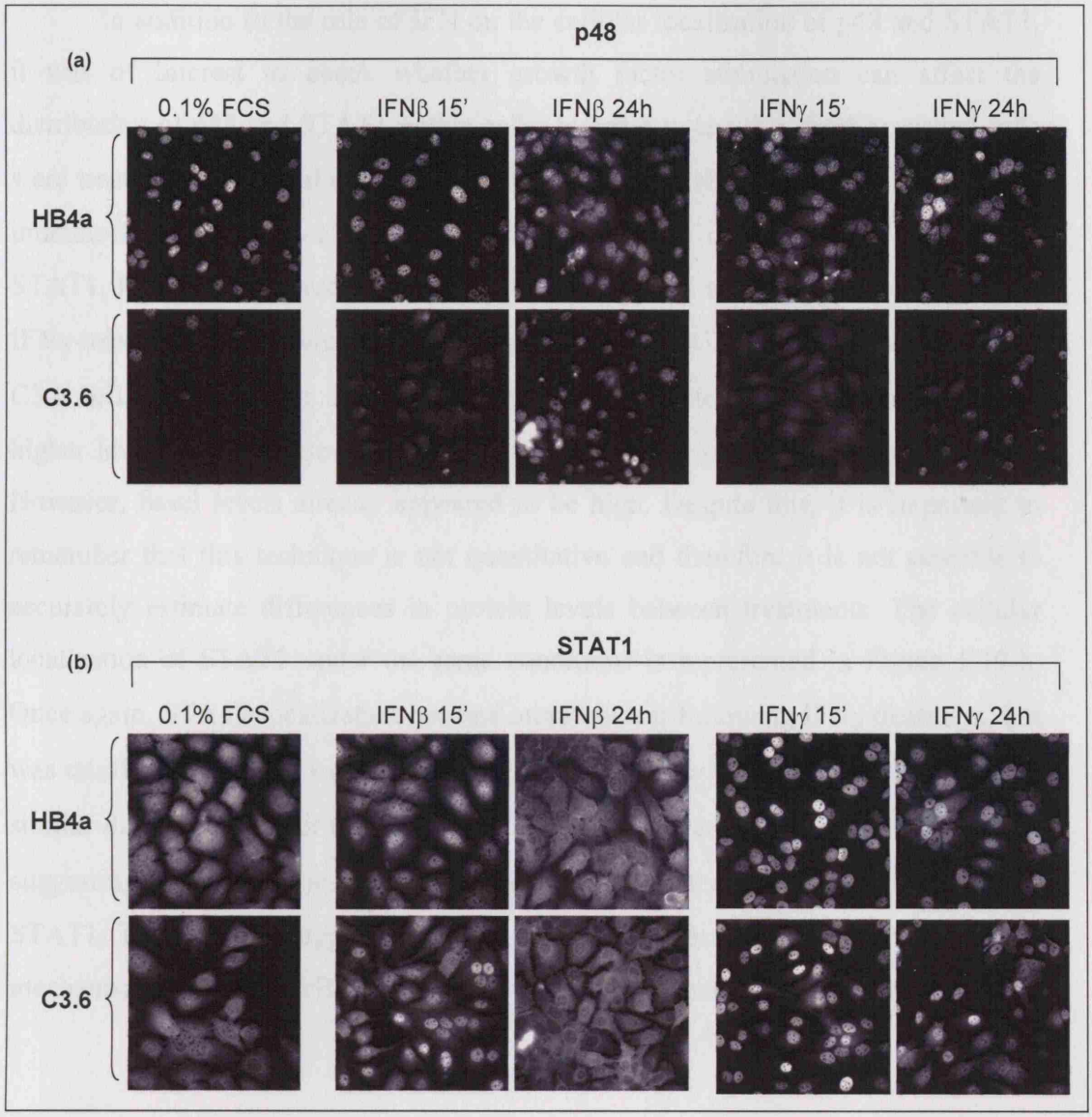

Figure 5.18: Cellular localization studies of p48 and STAT1 following IFN stimulation. HB4a and C3.6 cells were serum-starved for 24 hours and treated with IFNβ or IFNγ for 15 minutes or 24 hours. Immunofluorescence was performed as described above. **(a)** Staining pattern for p48 in HB4a and C3.6 cells following IFN stimulation. **(b)** Staining pattern of STAT1 under the same conditions as (a).

In addition to the role of IFN on the cellular localization of p48 and STAT1, it was of interest to check whether growth factor stimulation can affect the distribution of p48 and STAT1 within cells. In order to test this, freshly seeded cells were treated with EGF alone or in combination with IFNγ for 24h, and subjected to immunofluorescence as above to examine the pattern of distribution of p48 and STAT1. P48 remained nuclear under all conditions, and showed stronger staining in IFNγ-treated cells (Figure 5.19-a). Interestingly, in EGF and IFNγ co-stimulated C3.6 cells EGF did not appear to inhibit IFNγ-mediated upregulation of p48, as higher levels of p48 were observed when compared to serum-starved control cells. However, basal levels already appeared to be high. Despite this, it is important to remember that this technique is not quantitative and therefore it is not possible to accurately estimate differences in protein levels between treatments. The cellular localization of STAT1 under the same conditions is represented in Figure 5.19-b. Once again, STAT1 localization became more nuclear following IFNγ treatment, but was unaffected by EGF treatment. When EGF and IFNγ were used in combination, similar distribution patterns were seen as when cells were treated with IFNγ alone, suggesting that EGF does not affect the IFNγ-mediated nuclear translocation of STAT1. These results suggest that the regulation of subcellular localization is not a mechanism by which ErbB2 disrupts IFN signalling in these cell lines.

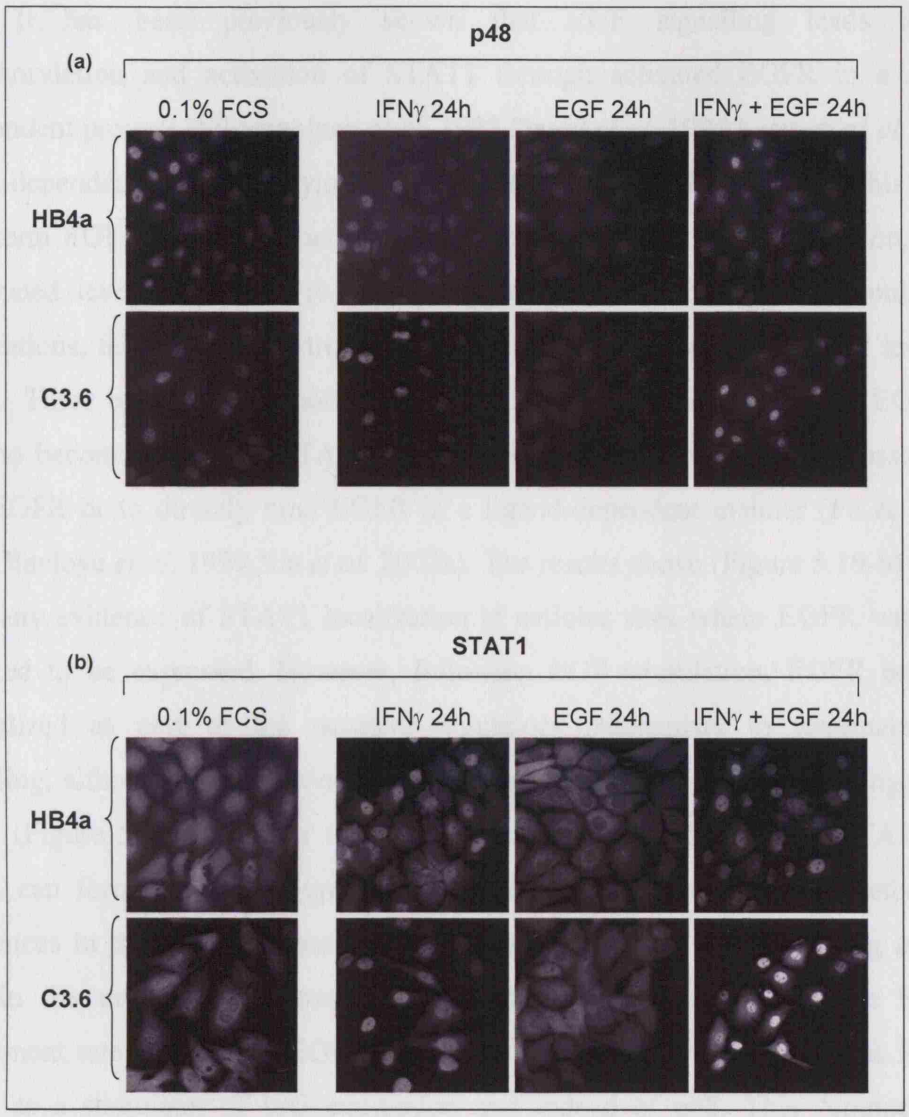

Figure 5.19: Effect of IFNγ and EGF co-stimulation on subcellular localization of p48 and STAT1. HB4a and C3.6 cells were serum-starved for 24h and then stimulated with IFNγ, EGF or IFNγ plus EGF for 24 hours. Cells were fixed and subjected to immunofluorescence for detection of p48 **(a)** and STAT1 **(b)**.

It has been previously shown that EGF signalling leads to the phosphorylation and activation of STAT1 through activated EGFR in a JAK1-independent process (Silvennoinen *et al.* 1993,David *et al.* 1996,Leaman *et al.* 1996) that is dependent on Src (Olayioye *et al.* 1999). In the cell lines used in this study, short-term EGF stimulation was also able to induce STAT1 phosphorylation, albeit at reduced levels compared to IFNs (Figure 5.10-a). Consistent with published observations, this effect was stronger in HB4a cells, which express higher levels of EGFR. There are various reports suggesting that STAT1 interacts with EGFR in order to become activated; STAT1 has been shown to be constitutively associated with EGFR or to directly bind EGFR in a ligand-dependent manner (Fu & Zhang 1993,Olayioye *et al.* 1999,Xia *et al.* 2002a). The results above (Figure 5.19-b) do not show any evidence of STAT1 localization at cellular sites where EGFR would be expected to be expressed. However, following EGF stimulation, EGFR becomes internalized as part of the negative regulatory mechanism to terminate EGF signalling, although STAT1 is no longer phosphorylated by EGF at the longer time points (Figure 5.10-a). It was therefore of interest to verify whether STAT1 and EGFR can form a complex upon short term EGF stimulation, and whether any differences in this binding pattern can account for reduced IFN signalling in C3.6 cells in the presence of growth factors. Moreover, differences in the STAT1 recruitment rate to activated EGFR may result in altered levels of available STAT1 to act as a stimulator of ISG expression and indeed of p48. This "competition" between the EGF and IFN signalling pathways could account for, at least in part, the inability of IFNγ to induce the expression of p48 in the presence of growth factor in C3.6 cells (Figure 5.13-a). To test this hypothesis, STAT1 immunoprecipitation studies were carried out on cell lysates of HB4a and C3.6 cells serum-starved or stimulated with EGF or HRG for 15 minutes (Figure 5.20-a).

There was no evidence of STAT1 binding to EGFR in either serum-starved or growth factor treated cells. Total cell lysates (TCL) from the same sample preps were also tested for EGFR and these showed that levels of expression were higher in HB4a than C3.6 cells, as previously observed (Timms *et al.* 2002). Cells were also stimulated with IFNβ or IFNγ for 15 minutes (Figure 5.20-b). Once again, there was no association of STAT1 to EGFR. In order to test whether immunoprecipitation can indeed provide information on the binding partners of STAT1, immunoprecipitates were also tested for STAT2. STAT2 was found to co-immunoprecipitate with

STAT1, although surprisingly this also occured in serum-starved cells. It is important to note that, despite higher levels of STAT1 in C3.6 cells, there was more STAT1/STAT2 complexe in the HB4a cells. Reverse experiments, where immunoprecipitates were prepared with EGFR antibodies and probed with STAT1 antibodies were carried out to further support these findings, but the antibodies used failed to immunoprecipitate EGFR. It will be interesting to further test the association of STAT1 with other EGFR-associated proteins, such as ErbB2, that may provide docking sites for STAT1 which may affect formation of the ISGF3 complex.

Figure 5.20: STAT1 immunoprecipitation studies. (a) HB4a and C3.6 cells were serum-starved for 24 hours and stimulated with EGF or HRG for 15 minutes. Cells were then lysed and 500ug of protein were mixed with protein A sepharose beads and STAT1-specific antibodies. This prep was then allowed to mix for 4 hours at 4°C on a gyrating wheel. Beads were then washed four times, re-suspended in sample buffer and boiled for 3 minutes before performing PAGE. A small amount of total cell lysate (TCL) was collected for each sample prior to immunoprecipitation to test for endogenous protein levels. **(b)** HB4a and C3.6 cells serum-starved and then stimulated with IFNβ or IFNγ for 15 minutes. Immunoprecipitation was carried out as in (a).

5.6.3 2D gel studies

Post-translational modifications are processing events that change the properties of proteins by proteolytic cleavage or by the addition of a modifying group to one or more amino acids, and can determine the protein's activity state, localization, turnover and interaction with binding partners. Although there have been a number of studies examining the transcriptional regulation of p48 (Weihua *et al.* 1997a,Weihua *et al.* 1997b,Roy *et al.* 2000,Hu *et al.* 2001b,Roy *et al.* 2002), very little has been reported about its post-translational modifications. For instance, it is not know whether p48 becomes phosphorylated or subjected to other modifications upon IFN stimulation or prior to binding to STAT proteins. Furthermore, while STAT proteins are known to be de-phosphorylated, targeted for degradation by ubiquitination (de Veer *et al.* 2001) or even "sumoylated" (i.e. covalently modified by the protein SUMO-1 to negatively regulate STAT activity) (Ungureanu *et al.* 2003), there are no reports on the fate of p48 after it has performed its role in inducing the transcription of ISGs.

2-dimensional gel electrophoresis (2DE) is a method of protein separation where proteins in a mixture are separated according to their isoelectric point (pI) in the first dimension (isoelectric focusing) and by molecular weight in the second dimension by standard SDS-PAGE (Gorg *et al.* 2000). 2DE often allows the visualization of different protein isoforms and can therefore be a useful tool in the study of protein post-translational modifications. 2DE experiments were therefore run to try and identify p48 isoforms that may provide clues to any possible post-translational modifications. HB4a and C3.6 cell lysates from serum-starved cells and cells treated with IFNγ alone or in combination with EGF for 24 hours were run on the 2D gels and then transferred onto PVDF membranes. Membranes were then probed using p48-specific antibodies. The results in Figure 5.21 showed a number spots that do not correspond to p48 and represent non-specific binding of the antibody. However, based on the known molecular weight and pI of p48 (MW. 43696, pI 5.6 – ExPASY database) it is possible to speculate which spots correspond to p48 (indicated by box). In addition, IFN stimulation increased the intensity of these spots, and this was inhibited in C3.6 cells by co-stimulation with EGF. These effects on protein level are identical to those previously seen in 1D immunoblotting experiments, suggesting that these spots are indeed p48.

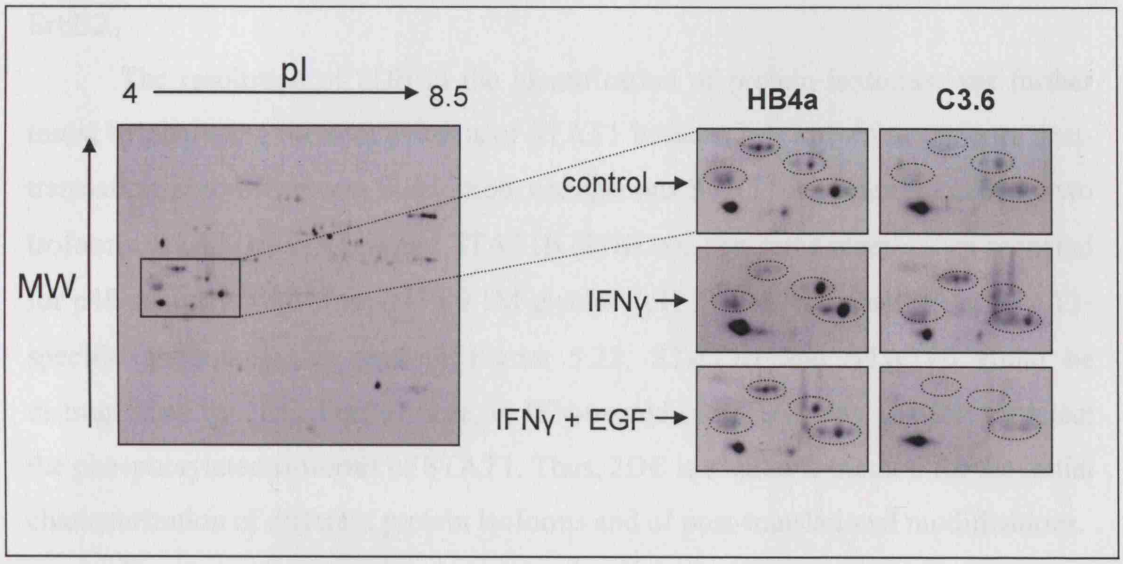

Figure 5.21: 2D-gel analysis of p48. HB4a and C3.6 cells were serum-starved for 24 hours and then stimulated with IFNγ alone or in combination with EGF for 24 hours. Cells were lysed in 2D-lysis buffer (see Materials & Methods). Protein samples (200 μg) were then applied to immobilized pH gradient (IPG) strips and left overnight at room temperature for strip re-hydration. Isoelectrofocusing was performed, followed by electrophoresis to separate proteins according to molecular weight. Proteins were then transferred onto PVDF membranes, which were blocked with 5% skimmed milk and subjected to immunoblotting using p48-specific antibodies. Spots believed to correspond to p48 are highlighted in the dotted circles.

Four sets of spots could be seen at the expected pI and molecular weight range of p48 which showed similar changes in expression; being barely detectable in serum-starved and EFG plus IFNγ treated C3.6 cells (Figure 5.21, indicated by dotted circles). This is indicative of possible post-translational modifications of p48. Whilst it is difficult to know what each of the putative p48 isoforms represent, they all appear to be co-regulated and so acute changes in post-translational modifications that affect pI or molecular weight do not appear to be involved in p48 regulation by ErbB2.

The resolution of 2DE in the identification of protein isoforms was further tested by analyzing the spot patterns of STAT1 because it is known to undergo post-translational modifications. In addition, endogenous STAT1 is present in cells in two isoforms, STAT1α (91KDa) and STAT1β (89KDa). The same membranes prepared for p48 analysis were stripped in 0.1M glycine (pH 2) and re-probed using STAT1-specific antibodies. As seen in Figure 5.22, STAT1α and STAT1β could be distinguished by 2DE. Furthermore, in IFN-treated samples it was possible to detect the phosphorylated isoforms of STAT1. Thus, 2DE is a suitable method for the initial characterization of different protein isoforms and of post-translational modifications.

Based on these results, it is speculated that p48 is comprised of different isoforms, suggesting that it undergoes post-translational modifications that may well affect its activity. These findings open a new array of hypotheses regarding the regulation of p48, and indeed its regulation by ErbB2 overexpression. It will be interesting to follow up on these results and carry out more detailed studies to further investigate the post-translational regulation of p48. However, no differences in post-translational modifications were observed that could suggest a mechanism of differential p48 regulation in ErbB2-overexpressing cells.

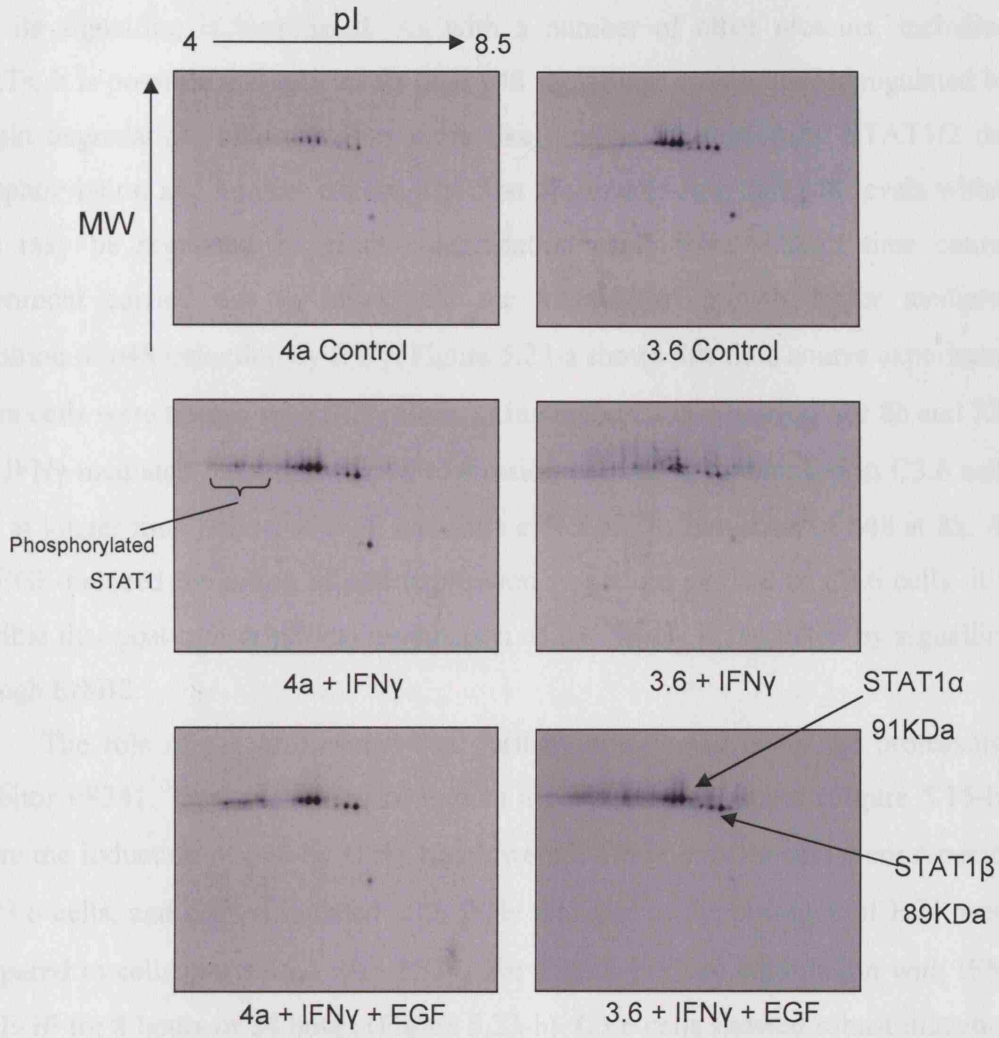

Figure 5.22: 2D-gel analysis of STAT1. PVDF membranes used in Figure 5.21 were stripped using 100mM glycine pH2 and re-probed using STAT1-specific antibodies. The two STAT1 isoforms, STAT1α and STAT1β are indicated, as is the change in spotting pattern thought to reflect STAT1 phosphorylation following IFN stimulation.

5.6.4 Cellular turnover of p48

As mentioned above, very little is known about the cellular regulation of p48. It is unclear at this stage what events follow the IFN-induced upregulation of p48 and how its signalling is terminated. As with a number of other proteins, including STATs, it is possible that p48 levels (and p48 signalling) are negatively regulated by protein degradation, although it is more likely to be terminated by STAT1/2 de-phosphorylation and nuclear export. The first clue suggesting that p48 levels within cells may be regulated by protein degradation came from a short time course experiment carried out to investigate the kinetics of growth factor mediated inhibition of p48 induction by IFNγ. Figure 5.23-a shows this time course experiment where cells were treated with IFNγ alone or in combination with EGF for 8h and 72h. The IFNγ-mediated induction of p48 expression seemed to be blocked in C3.6 cells only at longer time points, as EGF had little effect on the induction of p48 at 8h. As the EGF-induced inhibition of p48 expression was more marked in C3.6 cells, it is possible that post-transcriptional modulation of p48 levels is regulated by signalling through ErbB2.

The role of the proteasome was further investigated using the proteasome inhibitor PS341. This inhibitor was used in a previous experiment (Figure 5.15-b), where the induction of p48 by IFNγ was lowered. These experiments were repeated in C3.6 cells, and cells stimulated with IFNγ alone or in the presence of EGF were compared to cells pre-treated with PS341 for 1 hour prior to stimulation with IFNγ and EGF for 8 hours or 24 hours (Figure 5.23-b). C3.6 cells showed robust induction of p48 expression in response to IFNγ by 24 hours, and this was inhibited by co-stimulation with EGF. However, in cells pre-treated with PS341, IFNγ did not induce p48 expression to the same degree. Furthermore, randomly growing HB4a cells treated with PS431 for 24, 48 and 72 hours showed a time-dependent decrease in p48 levels (Figure 5.23-c). It is important to note, however, that these cells showed a level of toxicity to PS341 treatment and cell death was observed (data not shown). This could therefore account for some of the differences in protein expression observed. Nonetheless, these results indicate that proteasomal degradation plays a role in maintaining p48 expression.

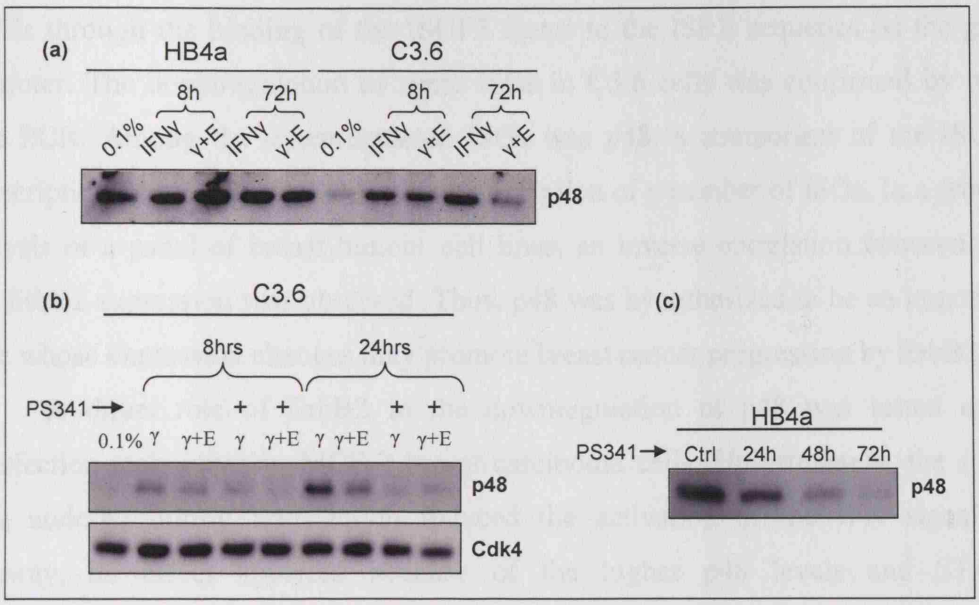

Figure 5.23: Proteasomal inhibition and p48 expression. (a) HB4a and C3.6 cells were serum-starved for 24 hours and then stimulated with IFNγ alone or in the presence of EGF for 8 hours or 72 hours. Expression levels of p48 were assessed using p48-specific antibodies. The delay in EGF-mediated downregulation of p48 suggests that growth factors can regulate p48 post-transcriptionally, possibly through a protein degradation pathway. **(b)** The role of the proteasome in p48 regulation was studied in C3.6 cells. Cells were pre-treated with the proteasome inhibitor PS341 for 1 hour and then stimulated with IFNγ +/- EGF for 8 or 24 hours. **(c)** Randomly growing HB4a cells were treated with PS341 for 24, 48 or 72 hours to test the effect of proteasome inhibition on p48 expression. PS341 was re-added every 24 hours to ensure continuous activity.

5.7 Chapter Conclusions & Discussion

In an attempt to identify potential markers of ErbB2-dependent breast cancers, microarray experiments were carried out in an ErbB2-overexpressing model cell system. A number of IFN-stimulated genes (ISGs) were found to be downregulated in the ErbB2-overexpressing cells, highlighting the possibility that a defect in this signalling pathway may play a role in malignant transformation. IFNs are known to play an important role in the negative regulation of cell proliferation through a number of mechanisms (Sangfelt *et al.* 2000). In order to further understand the mechanism of ErbB2-mediated cellular transformation, the IFN signalling pathway was further characterized in these cell lines. Clustering analysis of microarray data revealed that such downregulated ISGs were those that are primarily induced by type

I IFNs through the binding of the ISGF3 factor to the ISRE sequence on the gene promoter. The downregulation of some ISGs in C3.6 cells was confirmed by real-time PCR. Among the downregulated ISGs was p48, a component of the ISGF3 transcription factor responsible for the transcription of a number of ISGs. In a protein analysis of a panel of breast tumour cell lines, an inverse correlation between p48 and ErbB2 expression was observed. Thus, p48 was hypothesized to be an important gene whose expression changes may promote breast cancer progression by ErbB2.

A direct role of ErbB2 in the downregulation of p48 was tested using transfection techniques in MCF-7 breast carcinoma cells. Unfortunately, the stress cells undergo during transfection induced the activation of the IFN signalling pathway, an effect apparent because of the higher p48 levels and STAT1 phosphorylation in transfected cells compared to non-transfected cells. Although p48 levels were generally lower in ErbB2-transfected cells compared to cells transfected with the empty vector, it was not possible to establish a direct role of ErbB2 in the downregulation of p48 with a high degree of confidence. This finding has important implications on the study of gene function of IFN-related genes as well as other pathways. Transient transfection experiments are widely used in an attempt to better understand gene function and its corresponding role in cellular behaviour. The activation of the IFN signalling by this technique may well affect the cell behaviour and produce misleading results regarding a gene's function and the observed physiological response. Moreover, it is possible that such techniques can induce stress signalling pathways such as those involving JNK and p38, and this may alter cellular growth and proliferation.

Under the assumption that p48 is directly or indirectly downregulated by ErbB2, it was next necessary to establish a mechanistic link between the ErbB and the IFN signalling pathways. Despite expressing low levels of p48, C3.6 cells were shown to have an intact IFN signalling pathway, as p48 expression and STAT phosphorylation were induced in response to IFN stimulation, which resulted in a significant, dose-dependent, inhibition of cellular proliferation. While p48 protein expression was generally unaffected by growth factor stimulation on its own, it appears that induction of p48 by IFNγ can be overridden by growth factors; IFNγ-induced expression of p48 was blocked by co-stimulation of C3.6 cells with EGF or HRG. This effect was found to be ErbB receptor-dependent, as pre-treatment of cells with the ErbB kinase inhibitor AG1478 restored the ability of IFNγ to induce

maximal p48 expression in the presence of growth factors. When MTT proliferation assays were performed, IFNγ was able to inhibit growth factor-mediated cell proliferation more or less equally in both cell lines, whereas it was type I IFNβ that showed an inability to inhibit cellular proliferation in C3.6 cells in the presence of growth factors. This indicates that ErbB2 overexpression, possibly through its effect on p48 levels, can repress IFN signalling to provide cells with a proliferative advantage. Thus, it appears that ErbB2 overexpression can have a negative effect on the type II IFN-mediated induction of p48, which in turn is required for type I IFN-mediated inhibition of cellular proliferation. Indeed, IFNγ priming is thought to be an important mechanism of IFN signalling potentiation, since monocytes pre-treated with IFNγ have been shown to increase the magnitude of lipopolysaccharide (LPS) response (Hayes *et al.* 1995).

Various attempts were made to try and delineate a pathway whereby ErbB signalling could regulate IFN signalling and p48 expression. Cellular mechanisms including autocrine/paracrine signalling, altered cellular localization, complex formation and post-translational modifications were examined. Despite results showing evidence of autocrine/paracrine signalling, as demonstrated by the phosphorylation of STAT1 by conditioned media, no induction of p48 was observed and no differences between the normal and ErbB2 overexpressing cell lines were seen, although longer time points were not examined. Moreover, no basal secretion of IFNs was observed in these cell lines (Prof. Ian Kerr, personal communication). Similarly, there were no differences in the cellular localization of p48 or STAT1 observed between the two cell lines and the intensity of cell staining pattern was consistent with immunoblotting data for p48 levels and STAT1 phosphorylation and re-localization to the nucleus. There were no unexpected changes in cellular location when cells were co-stimulated with growth factors. This data shows that cellular re-localization is unlikely to be a mechanism by which ErbB2 signalling controls p48 and IFN signalling. Moreover, immunoprecipitation studies showed that STAT1 does not bind to EGFR despite it being activated by EGF, suggesting that STAT1 availability is not a contributing factor to the regulation of p48 expression. 2DGE studies showed evidence of multiple isoforms of p48, suggesting that in addition to the relatively well studied transcriptional regulation, p48 activity may be regulated

by post-translational modifications such as phosphorylation or ISGylation (Malakhov *et al.* 2003).

Additional experiments performed showed evidence of a role for the proteasome in the regulation of p48 cellular turnover. The data suggests that, rather than directly inhibiting the transcriptional activation of p48, EGF is actually inducing its downregulation through other mechanisms, such as by promoting its degradation. Surprisingly, when the proteasomal inhibitor PS341 was used to pre-treat C3.6 cells, p48 induction by IFNγ was inhibited. In addition, stimulation of HB4a cells with PS341 on its own caused a decrease in the levels of p48, an effect opposite of that expected if p48 was being degraded by the proteasome in response to growth factor. These results are interesting because they add yet another layer of complexity to the regulation of p48 and its possible role in cell signalling. Although these experiments do not provide sufficient amounts of data to draw a definitive mode of p48 regulation, they provide a clue to new directions in the study of the activity and regulation of this protein. Based on the results presented in this Chapter, a number of potential mechanisms of p48 regulation are proposed. These are discussed in Chapter 6.

In conclusion, the work carried out in this chapter extends the findings obtained using microarray technology to further characterize an important signalling pathway that may significantly contribute to the development of ErbB2-related breast cancers. Importantly, the findings presented here may be relevant to *in vivo* tumour biology since IFNγ is increasingly recognized as an important molecule that promotes protective host responses to tumours, and it has been shown to prevent the development of primary and transplanted tumours (Ikeda *et al.* 2002). Moreover, although IFNs are used routinely in the treatment of cancers including multiple myeloma, chronic myelogenous leukemia, hairy cell leukemia, and malignant melanoma, some are not responsive to such treatment. It is important to understand the mechanisms of such resistance and, based on scientific data obtained through studies like the one presented here, devise therapies with improved efficacy.

Chapter 6: DISCUSSION & FUTURE PROSPECTS

Receptor tyrosine kinases (RTKs) are primary mediators of signals that can determine the fate of cells by interpreting growth-regulatory messages, activating intracellular signalling pathways and ultimately altering gene expression and protein synthesis (Holbro & Hynes 2004). These receptors play an essential role in the regulation of cell differentiation, proliferation, apoptosis, growth and migration. RTK activation is mediated through growth factors which bind the extracellular domain of the receptor, resulting in receptor dimerization and activation through the phosphorylation of tyrosine residues in the receptor's cytoplasmic domain. This in turn creates docking sites that result in the recruitment of various Src homology 2 (SH2) or PTB domain-containing adaptor proteins and enzymes to specific locations in the receptor, thereby linking RTKs to a variety of intracellular signalling pathways (Schlessinger 2000). The most well-characterized signal transduction pathways that mediate the downstream effects of RTK activation are the Ras-Raf-MAP kinase pathway, the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and the JAK/STAT signalling pathway (Bell & Ryan 2005). The activity of RTKs is tightly controlled through a number of mechanisms, which include its dephosphorylation by protein tyrosine phosphatases (Zhang *et al.* 2002), receptor removal from the cell surface via ligand-induced receptor endocytosis (Waterman & Yarden 2001) and/or receptor ubiquitination followed by its degradation (Shtiegman & Yarden 2003). Indeed, this regulatory capacity is of critical importance, since errors in these control mechanisms can result in a variety of diseases, including cancer, through increased receptor activity and consequential enhanced tumour-promoting activities.

The ErbB family of RTKs is comprised of EGFR, ErbB2, ErbB3 and ErbB4. No ErbB2-specific ligands have been identified to date, and ErbB3 lacks intrinsic tyrosine kinase activity. Thus, these receptors rely on dimerization to other ErbB receptors to become activated and for the initiation of signal transduction events. De-regulated expression and/or function of ErbB receptors through several mechanisms, including receptor overexpression, gene amplification, activating mutations, overexpression of receptor ligands and/or loss of their negative regulatory mechanisms, have been implicated in a number of human cancers. Thus, ErbB receptors are important targets for cancer therapy, and a number of agents, which are

either monoclonal antibodies directed to the receptor's extracellular domain or small molecule ATP-competitive inhibitors of the receptor's tyrosine kinase activity, have been described (Baselga & Arteaga 2005).

The ErbB2 receptor was shown to be overexpressed in 25 to 30% of invasive breast cancers due to gene amplification (Slamon *et al.* 1987) and this receptor represents an important target in breast cancer therapy. Indeed, the humanised monoclonal antibody targeted against ErbB2, Herceptin (trastuzumab) (Carter *et al.* 1992), has already been approved for clinical use to treat patients with breast cancer (Horton 2002), and a number of other therapies against ErbB2 (and other ErbB receptors) are under development (Gschwind *et al.* 2004). However, the exact role of ErbB2 overexpression in the development of cancer and the mechanisms by which Herceptin inhibits specific growth of ErbB2 overexpressing cells *in vivo* are not well understood. The signalling pathways activated downstream of ErbB2 are complex as multiple ligands can trigger different dimer combinations between ErbB family members, and importantly ErbB2 is the preferred partner in these dimerization/activation events (Graus-Porta *et al.* 1997). In addition, cross-talks between signalling pathways can also occur, therefore generating a highly complex network of signals. This has the potential to activate and repress the expression of a diverse set of genes that ultimately determines the functional status and fate of a cell.

The aim of this project was to investigate the effects of ErbB2 overexpression and ligand-induced signalling on the gene expression profile of a model cellular system of breast cancer in order to identify new components of this complex machinery, and ultimately to identify breast cancer biomarkers for further targeted therapies. This was achieved by comparative microarray analysis of the human mammary luminal epithelial cell (HMLEC) line HB4a and its ErbB2-overexpressing derivative, C3.6, under serum-starved conditions as well as after a time course treatment with EGF or HRG, EGFR- and ErbB3-specific ligands, respectively. These luminal cell lines are the appropriate cell type to use, since most breast cancers arise from luminal epithelial cells. In addition, C3.6 cells overexpress levels of ErbB2 similar to that seen in carcinomas (Harris *et al.* 1999). Thus, this cellular system is a good model for the study of ErbB2-dependent breast cancer and is likely to represent the early stages of transformation. Note, however, that the ErbB2 amplicon (17.12-q21), which could be heterogeneous in copy number and associated mutations, is not represented in these cell lines, and therefore this study addresses gene expression

changes resulting exclusively from changes in ErbB2 levels rather than by co-amplified or altered genes, which may represent an important factor in the transformation of the breast. Indeed, analysis of the amplicon structure in 330 breast tumours showed that a common region of amplification containing at least 10 transcribed sequences, including the adaptor protein Grb7 which maps close to ErbB2, is frequently overexpressed in concert with ErbB2 and these may therefore be involved in tumour generation (Kauraniemi *et al.* 2003).

Microarray technology is a powerful tool for large-scale gene expression studies because it allows the measurement of comparative expression of thousands of genes in a single experiment. The method relies on the principle of nucleic acid hybridization between labelled free targets derived from the biological sample RNA molecules and large arrayed sets of DNA fragments corresponding to specific genes which are spotted onto a solid support. The amount of labelled cDNA target that hybridizes with its corresponding probe is proportional to its abundance in the original RNA sample, and profiles for different samples can then compared to each other (Duggan *et al.* 1999). Microarray technology has already been proven to be a useful tool in the study of breast cancer, and was also shown to be able to distinguish tumours with different clinical status (e.g. oestrogen receptor positive and negative tumours (West *et al.* 2001) and tumours bearing BRCA1 or BRCA2 mutations (Hedenfalk *et al.* 2001)), to predict patient prognosis (van de Vijver *et al.* 2002,van't Veer *et al.* 2002) and to discriminate tumours that are not responsive to chemotherapy (Sotiriou *et al.* 2002) based on gene expression patterns. In addition, a number of candidate markers of breast cancer have been identified using microarrays (Lacroix *et al.* 2002). Thus, microarrays analysis can provide important information regarding the gene expression status of tumours and may help in the identification of novel markers of breast cancer.

6.1 Summary of Microarray Data Analysis Findings

The microarray analysis presented in this thesis was used to assess the effect of ErbB2 overexpression on the gene expression profile of HMLECs for the identification of potential markers of ErbB2-dependent signalling and breast cancer. In addition, microarrays were used to analyse changes in gene expression associated with EGF and HRG signalling in the HB4a and C3.6 cells to define the specificity of

signalling through EGFR and ErbB3 and to test the effects of ErbB2 overexpression in these signalling events. A reference experimental design was used, whereby each sample was co-hybridized with a common reference sample to allow cross-comparison of all the conditions studied (two cell lines, two growth factors and four time points, giving a total of 14 conditions). SAM (Significance Analysis of Microarrays) software with a FDR of 3% (Tusher *et al.* 2001) was then used to identify significant changes in gene expression. A total of 775 genes were found to be changing in one or more conditions and as shown in Figure 3.8-a. 309 genes were differentially expressed in the ErbB2 overexpressing cells (C3.6) when compared to parental cells (HB4a), 172 of which were not responsive to growth factor stimulation. 199 genes changed only in response to EGF, whereas 269 genes were responsive only to HRG and 135 genes were responsive to both growth factors. A complete list of the 775 changing genes is shown in Appendix A.

The genes identified as differentially expressed are known to be involved in a variety of cellular processes that have been linked to tumour development, growth and metastasis. These included:

(i) cell adhesion and/or motility genes, which generally displayed growth factor responsiveness rather than ErbB2-dependent expression, although changes in the expression of these genes were genrally significant and more robust in the C3.6 cells. These genes included vimentin, zyxin, vinculin, transgelin, L-plastin, tubulin α1 and β2, actin β, the integrin subunits α2, α3, β4 and β1 (represented by the clone 343072_A), integrin β4 binding protein, laminin α3, annexins 1 and 2, fibronectin 1, cadherins 3 and 13 and the keratins 6, 13 and 15. As cells progress through a sequence of steps in the metastatic process and must change their adhesion and motility properties in order to successfully complete these steps and form metastases at distant sites (Chambers *et al.* 2002). Thus, the identification of these genes is an important finding that suggests that ErbB signalling can transcriptionally regulate their expression and may have implications in tumour metastasis. Adhesion assays performed in this laboratory further confirmed that ErbB2 overexpression can alter the adhesive properties of the C3.6 cells, and these cells were found to be significantly less adhesive than the HB4a cells (White *et al.* 2004). Notably, fibronectin could restore this adhesion showing that loss of fibronectin gene expression may be an important step in the metastatic process;

(ii) growth factors (VEGF, AREG, PDGFB), growth factor-related genes (IGFBP3, IGFBP5) and growth factor and other receptors (ErbB2, ErbB3, gp130/oncostatin M receptor, uPAR), suggesting that there is an important role for autocrine/paracrine signalling in ErbB2-mediated signalling and transformation. Media swap experiments using conditioned media from HB4a and C3.6 cells showed that, in these cells, growth factor stimulation can indeed result in autocrine/paracrine activation of ErbB-dependent signal transduction pathways (Figure 4.5);

(iii) genes that regulate cellular proliferation and cell cycle progression, such as E2F4, CDKN1A (p21), CHEK1, CKS2, SFN (14-3-3σ), PHB, Myc, GADD45 and S100P, whose altered expression have obvious implications in ErbB-mediated cellular proliferation;

(iv) signalling kinases (MEK1, MEK3, MAP4K4, CK1α and ε, S6K2) and phosphatases (PPP1CC/PPP1CA, PPP2R4, PPP2R1, PTP4A1, PTP4A2, DUSP1 and 5), showing that positive and negative regulators of important signal transduction pathways are transcriptionally regulated by ErbB signalling;

(v) genes involved in ubiquitin (UBC, UBE2D2, UBE2N, UBE2L6 and USP14) and ubiquitin-like (G1P2, UBL1) mediated protein modification, suggesting that de-regulated protein degradation may be an important mechanism of transformation through the lack of negative signalling regulation, although further studies are required to confirm this;

(vi) genes involved in regulation of cellular redox status (CYBA COX6C, COX8, PRDX1 and 5, GSTP1, MGST1), suggesting that ErbB2 overexpression may affect the cellular response to oxidative stress through the regulated expression of redox-sensitive enzymes and stress-induced genes. These findings are interesting because similar findings were obtained in parallel redox proteomics experiments carried out in our laboratory (Chan *et al.* 2005), where it was shown that redox signalling was affected by ErbB2 overexpression;

(vii) IFN-responsive genes, all of which were downregulated in C3.6 cells and showed little or no growth factor responsiveness (p48, OAS1, PRKR, G1P2, IFITM1, IFITM2, IFIT1). IFNs are negative regulators of proliferation, and therefore these findings suggest that downregulation of the IFN signalling pathway may represent an ErbB2-mediated mechanism of increased cellular proliferation. This hypothesis is further discussed below;

(viii) a number of clones with no gene annotation by the chip manufacturer were identified in this analysis as being differentially expressed. As discussed in Chapter 3 (Section 3.4.4), BLAST analysis for some of these clones revealed that their sequences do indeed match known genes, although many of these have very little or no functional annotation. Future follow up work on these genes may lead to the identification of novel, uncharacterized targets of ErbB2 overexpression and ErbB signalling which will further increase our understanding of the role of ErbB2 in breast cancer progression.

Genes that were differentially expressed in the ErbB2-overexpressing cells under serum-starved conditions are shown in Table 3.2. 40 genes were upregulated and 21 were downregulated. These genes are likely to be direct targets of ErbB2 overexpression, as their transcription is probably regulated by the ligand-independent constitutive signalling associated with ErbB2 overexpression (Harari & Yarden 2000). Some of these genes have been previously associated with malignant transformation of the breast, such as the overexpression of S100P (Guerreiro, I *et al.* 2000), EMP1 (Lacroix *et al.* 2002), LCP1 (Lin *et al.* 1988b,White *et al.* 2004), annexin 2 (Schwartz-Albiez *et al.* 1993) and the downregulation of CYBA (Powell *et al.* 2002) and IGFBP3 (Mackay *et al.* 2003,White *et al.* 2004). Annexin 1 was downregulated in C3.6 cells, but its role in breast cancer is less clear since both its up- and downregulation have been reported in breast cancers (Pencil & Toth 1998,Shen *et al.* 2005). In addition, the prohibitin gene, which is thought to be a tumour suppressor (Wang *et al.* 1999) was upregulated in C3.6 cells, contradicting reports that this gene is often mutated (resulting in gene inactivation) in breast cancers (Sato *et al.* 1993).

Genes not previously linked to ErbB2-dependent breast cancers were also identified in this analysis. One of these genes was NEDD9 (enhancer of filamentation 1, HEF1), which was found to be upregulated in C3.6 cells. This gene codes for a docking protein which, through multiple protein interactions at focal adhesion sites, activates signal transduction pathways in response to integrin receptor binding to extracellular matrix (Law *et al.* 1998). NEDD9 has been shown in induce apoptosis of human mammary cells when overexpressed (Law *et al.* 2000), but its regulation by ErbB2 has not been previously documented. Another novel finding was the downregulation of the stress-inducible gene SERPINH1 (HSP-47) in the ErbB2-overexpressing cells. This gene is thought to be a collagen-specific molecular

chaperone and is involved in collagen processing and/or secretion under normal conditions (Nagata 1996b), but once again no regulation by ErbB2 has been reported. Copine III, which was upregulated in response to HRG and overexpressed in C3.6 cells, showed a similar pattern of protein expression (Gharbi *et al.* 2002). The copines are thought to bind to phospholipids through their two C2 domains and have therefore been proposed to play a role in vesicular trafficking (Creutz *et al.* 1998). The copines also contain a C-terminal A-domain that is found in the extracellular portion of integrins enabling them to bind extracellular matrix proteins (Lee *et al.* 1995a) and copine III is reported to have an associated kinase activity (Caudell *et al.* 2000). This poorly characterized protein therefore represents an interesting target to follow up on.

6.1.1 Differential signalling properties in the ErbB2-overexpressing cells in response to growth factor stimulation

Based on the differential expression levels of ErbB receptors in the HB4a and C3.6 cells (Figure 3.1 and (Timms *et al.* 2002)), it is possible to hypothesize which receptor pairs are responsible for signal transduction in response to EGF and HRG. HB4a cells express relatively high levels of EGFR and therefore EGF is expected to induce signalling primarily through EGFR/EGFR homodimers in these cells. However, it is also possible that EGFR/ErbB2 and EGFR/ErbB3 heterodimers are activated by EGF, particularly in the C3.6 cells where ErbB2 and ErbB3 are highly expressed (Figure 3.1). HRG, on the other hand, binds to ErbB3 and ErbB4. ErbB4 is not expressed in the cell lines used here, and ErbB3 has no intrinsic kinase activity, and therefore must form heterodimers in order to activate signalling. Since ErbB2 is overexpressed in the C3.6 cells and it is the preferred dimerization partner for the other ErbB receptors, it is expected that HRG will induce signalling exclusively through ErbB2/ErbB3 dimers in the C3.6 cells but through EGFR/ErbB3 dimers in the HB4a cells. The differences in receptor levels between the HB4a and C3.6 cells are likely to have important implications in ErbB2-dependent cellular transformation and it is possible that ErbB2 has modulated the expression of EGFR and ErbB3 to bias receptor dimer formation towards a more potent receptor complex. Indeed, ErbB2/ErbB3 dimers have been shown to induce the most potent proliferative signals (Pinkas-Kramarski *et al.* 1996), so the high levels of ErbB3 seen in the C3.6 as a

consequence of ErbB2 overexpression would in turn result in the higher proliferative capacity of these cells (Timms *et al.* 2002), altered adhesion (White *et al.* 2004) and motility and survival.

An important observation in the present microarray analysis was the inability of a number of genes to respond to HRG in the HB4a cells, while being clearly responsive in the C3.6 cells (Figure 3.16, sub-clusters ii and iii). Genes upregulated by HRG in C3.6 cells but not in HB4a cells included the proto-oncogene MYC and the MYC-regulated gene EMP1, the MAPK MAP2K3, the regulators of MAPK signalling DUSP1 and DUSP5, the growth factor PDGFB and SERPINE1, which has recently been identified as a breast cancer prognostic marker (Harbeck *et al.* 2004). All of these genes are involved in the regulation of cellular proliferation and/or survival and therefore are likely to influence the HRG-induced proliferation of ErbB2-overexpressing breast cancer cells. Another important finding was the different expression pattern of genes regulated by EGF compared to HRG-regulated genes. Typically, EGF-responsive genes showed a higher level of induction but were generally only transiently changing, while HRG genes showed lower fold-changes but the changes in expression were longer lived (see Figure 3.19 compared to Figure 3.21). Once again, this points to a role for increased HRG-induced proliferation and survival in ErbB2-overexpressing cells. Collectively, these findings demonstrate that ErbB2 overexpression can alter the cell's signalling properties, possibly through the modulation of the other ErbB receptors' expression, and increase their proliferative capacity, survival and adhesion to promote tumour formation.

6.1.2 Microarray analysis of ErbB2-dependent changes in gene expression: comparison with current literature & data reproducibility.

Microarray experiments have been carried out using cell lines and tumour samples to try and identify differences in gene expression patterns between ErbB2-negative and ErbB2-positive breast tumours. The studies by Mackay *et al* (Mackay *et al.* 2003) and White *et al* (White *et al.* 2004) used the same cellular model of ErbB2 overexpression and therefore immediately relevant to the study presented here. The results from these studies and the similarities to the results found here were discussed in Chapter 3. Bertucci *et al* (Bertucci *et al.* 2004) analysed the expression profile of

145 randomly chosen breast cancer samples to identify an ErbB2 "gene expression signature" comprised of 36 unique sequences, 29 of which were well-characterized genes. Based on the ErbB2 signature, an independent series of new samples (54 breast tumours and 16 breast cancer cell lines) was found to be successfully classified according to their ErbB2 immunohistochemistry status, with only one misplaced sample. Genes differentially expressed in ErbB2-positive tumours included six genes that are located within the ErbB2 amplicon on region 12q of chromosome 17 (GRB7, PPARBP, PSMB3, RPL19, PPP1R1B and NR1D1). None of these genes were significantly changing between C3.6 and HB4a cells. PSMB3, a subunit of the 20S core structure of the proteasome, was present in the 775 genes identified here, but it was found to be only responsive to HRG stimulation. Other genes in the ErbB2 gene expression signature included OAS2, CDH15, GATA4, MAP2K6, CSTA, and three integrins (ITGA2, ITGA2B and ITGB3). With the exception of CSTA (cystatin A) and ITGA2, none of the genes in the ErbB2 signature were found in the present analysis, although other family members were present among the 775 genes found here. Other groups have reported a co-amplification of genes in the ErbB2 amplicon (Kauraniemi *et al.* 2001,Sorlie *et al.* 2003,Willis *et al.* 2003), indicating that genes located in the vicinity of ErbB2 are frequently co-upregulated following DNA amplification. Similar to the current study, the study by Mackay *et al*, using C3.6 and C5.2 cells did not find any of the genes on the ErbB2 amplicon. These findings are not surprising given that, as previously discussed, these cell lines do not contain the ErbB2 amplicon.

An important microarray study of breast cancer was performed by van't Veer *et al* (van't Veer *et al.* 2002). Using cRNA derived from 98 primary breast cancer patients, they were able to group together tumours based on their gene expression patterns and with that to distinguish between "good prognosis" and "bad prognosis" patients. By correlating the expression of each gene with disease outcome, they identified 231 genes that were significantly associated with disease outcome. The expression data was further statistically manipulated until they identified 70 marker genes that could successfully predict patient prognosis based on their expression pattern. This classifier set predicted correctly the outcome of 83% of patients. A follow-up study proved the efficiency of this classifier as a survival predictor on a large set of 295 tumour specimens (van de Vijver *et al.* 2002). Of these 70 marker genes, only three genes were identified in the present analysis. These are GMPS,

IGFBP5 and PRC1. Twelve other genes identified here were not exact matches with the genes within the 70-gene classifier, but were members of the same gene families. Examples include SERF2 (SERF1 in the classifier), RAB1B and RAB7 (RAB6B in the classifier), GSTP and GSTO (GSTM1 in the classifier) and MCM5 (MCM6 in the classifier). Interestingly, none of the classic genes associated with breast cancer disease outcome, such as ErbB2, c-myc and ER-α, were present in the marker set of 70 genes.

Sorlie *et al* (Sorlie *et al.* 2001) analysed the gene expression profiles of 78 breast carcinomas and, using hierarchical clustering techniques of 456 genes, were able to assign breast tumours to one of five different subtypes, each with a distinctive expression profile. They identified a cluster of ErbB2-positive tumours that contained ErbB2 itself and other co-regulated genes that included genes present in the ErbB2 amplicon, such as GRB7. Based on their expression profiles, tumours assigned to the ErbB2-positive subtype were associated with the shortest patient survival time. None of the genes present in the ErbB2-positive cluster, which included TNFR and TGFB1, were identified in the present study. Importantly, the sets of survival related genes identified by these two studies had only a few genes in common – of the 456 genes of Sorlie *et al*, only 17 also appeared in the 231 of van't Veer *et al.* Another study by Ramaswamy *et al* (Ramaswamy *et al.* 2003) identified a set of 128 genes that could separate metastases from primary tumours. A refined set of 17 metastasis-associated genes was tested in a diverse set of primary tumours, including 78 small stage I primary breast adenocarcinomas. They showed that tumours bearing the gene expression signature at diagnosis were more likely to develop distant metastasis than those lacking the signature. Once again, the similarity between datasets was very small, with only two genes being shared between the set of Sorlie *et al* and Ramaswamy *et al.*

In an attempt to explain the inconsistency between lists of survival-related genes derived from different experiments, Ein-Dor *et al* investigated the breast cancer dataset from van't Veer *et al* (Ein-Dor *et al.* 2004). They found that many sets of 70 genes, not just the top ranking ones described by van't Veer *et al*, can be used to predict survival and therefore the 70 genes selected by van't Veer cannot be considered as the main candidates for targeting anti-cancer treatments. In addition, they showed that the relative ranking of genes on the basis of correlation with survival, changes drastically when different training sets are used. Thus, although

studies like the examples above may be useful for predicting patient prognosis, they may not necessarily represent the best method for identifying markers of breast cancer that could be used as therapeutic targets.

In conclusion, the study presented here allowed the identification of a number of potential markers of ErbB2 overexpression as well as genes involved in signalling pathways downstream of ErbB receptors. However, as exemplified above, many microarray results are not reproduced across different studies. In this regard, an important consideration is that similar underlying biological processes can be expected to differ across organisms, genetic variants and environmental or other external conditions. Thus, microarray experiments are a valuable tool to identify potential markers of breast cancer, but further work must be carried out to ensure these are genuine and reproducible markers of breast cancer progression to allow confident diagnosis and treatment of this disease. Finally, it is important to note that the microarrays used in the present study do not contain the complete set of genes in the human genome, representing only about 6,000 unique genes. Since the beginning of this study, the Sanger Centre has produced a new batch of arrays containing a redundant set of about 15,000 clones. Other commercial array manufacturers are currently able to produce arrays containing a very large number of genes, and examples include Agilent Technologies, which uses ink-jet technology to produce onligonucleotide arrays containing up to 41,000 genes and transcripts[†††], and Affymetrix, which produces oligonucleotide microarrays containing over 54,000 probe sets and 38,500 well-characterized human genes[‡‡‡].

6.1.3 The need for data validation

The large number of variables compared in the present analysis made the analysis of this data a challenging process. Microarray data analysis of time course experiments is normally complicated because of the complex nature of gene regulatory networks, where the expression of one gene may affect the expression of another at a later time, although specific data analysis methods have been described for analysis of such time-dependent data (Li *et al.* 2002a). The study presented in this

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[†††] http://www.chem.agilent.com/temp/rad5B113/00048373.pdf

[‡‡‡] http://www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf

thesis is even more complicated because we wished to simultaneously compare and contrast four parallel time course experiments (using two distinct growth factors in two different cell lines). It was therefore decided to carry out pair-wise comparisons across each of the 14 conditions being studied to identify statistically significant ErbB2-depedent and EGF- or HRG-dependent changes in gene expression separately. Note that, because of the pair-wise fashion of data analysis, a given gene in the final list generated can show a significant change in one or more of the pair-wise comparisons. Thus, not all points in the expression graphs shown in this thesis may represent significant changes.

In addition, despite best efforts to quality control images and to normalize and quantitate fluorescent intensities, the microarray data obtained here had generally poor reproducibility between replicates. Reproducibility problems in microarray experiments can result from random errors and systematic errors, or biases. There are many sources of systematic variation in cDNA microarray experiments which affect the measured gene expression levels, including quality of starting material, differences in print tips, spatial location of spots and dye-biases, which can stem from a variety of factors such as efficiency of dye incorporation, experimental variability in hybridization and processing procedures, or scanner settings at the data collection step. This variability between replicates complicates the data filtering process, and it is possible that a number of significantly changing genes may have been filtered out during statistical analysis. Moreover, genes found to be significantly changing must be further investigated to ensure that the changes observed are indeed representative of the biological process being investigated.

The above observations highlight the need for data validation using different experimental assays, such as quantitative real time PCR (qRT-PCR). This technology has become one of the most widely used methods of gene quantitation because it has a large dynamic range and high sensitivity as well as accurate quantification. In this technology, the use of gene-specific fluorescence probes enables the amplification process to be monitored in real-time, and the initial concentration of a given gene target can estimated based on the amplification profiles. Initial copy numbers of mRNA targets are quantified during real-time PCR analysis based on a comparison with a threshold cycle (Ct), defined as the cycle at which fluorescence is determined to be above background signal and the PCR product is increasing exponentially (Giulietti *et al.* 2001). The advantage of qRT-PCR over conventional PCR is that it

allows the quantification of the target gene at a point where the synthesis of PCR product is limited only by the amount of starting material rather than by other factors such as decreasing primer concentrations and enzyme stability, as is the case for end-point analysis (Bustin 2000).

Unfortunately, there is currently no method available to validate large-scale gene expression data such as the one presented here and when cost and time are important issues, a smaller sample set must be chosen for further studies. Here, 14 genes were chosen for further validation by qRT-PCR, and the findings are briefly summarized below.

6.2 Validation of Microarray Data

Fourteen genes were chosen for validation by qRT-PCR. These were: amphiregulin (AREG), cathepsins B and C (CTSB and CTSC), insulin-like growth factor binding protein 3 (IGFBP3), S100P, vascular endothelial growth factor (VEGF), ezrin (VIL2), vimentin (VIM), zyxin (ZYX), 14-3-3σ (SFN) and the IFN-related genes p48, STAT1, OAS1 and G1P2. All genes, with the exception of VIM and CTSB, showed good qualitative, although not quantitative, correlation with the results obtained by microarray. The reasons for these discrepancies are not known, but possible explanations include non-specific amplification of splice-variants, or non-specific hybridization of other family members onto the arrays during hybridization, thereby affecting the fluorescence intensity signals and relative quantitation of expression. The expression of an additional three IFN-responsive genes not identified in the microarray was also analysed by qRT-PCR. As with the other IFN-related genes, they were expected to be downregulated in C3.6 cells and indeed this was the case. One of the genes, USP18 was not represented on the array, while the other two, SOD2 and UBE1L, were present on the array but failed to pass statistical tests during data filtering. These results suggest that indeed data variability can lead to false negative results and highlight the need for careful interpretation of the data.

As mentioned above, the pattern of gene expression was similar between the two platforms, but the actual quantitative values were often many-fold higher in the qRT-PCR experiments compared with the microarray experiments. Thus, it is likely that arrays underestimate the real expression change as detected by qRT-PCR.

Rajeevan *et al* (Rajeevan *et al.* 2001) have also compared gene expression data obtained using microarrays and qRT-PCR. Similar to the findings presented here, these investigators found that the majority of array results were qualitatively accurate (71%), but there were significant quantitative differences between array- and qRT-PCR-based data, with 10 of the 14 genes validated by qRT-PCR showing greater expression differences than that determined by microarray. As previously discussed, cross-hybridization of genes with high sequence similarities between family members may mask true expression differences. Another confounding factor in the quantitative analysis of microarray data arises from low abundance mRNAs, which can result in low intensity spots. This can affect relative quantitation of gene expression, particularly if there is a high level of background on the array slide. Moreover, arrayed DNAs may produce non-specific background signals during the experiment as a result of repetitive elements such as poly(A) tails and common motifs. These observations suggest that differences in the sensitivities between the two methods exist, and highlight the need for the development of uniform validation methods which enable more accurate gene expression quantitation.

Where antibodies were available, mRNA data obtained from microarray experiments were correlated with protein expression by immunoblotting. Parallel proteomics experiments carried out in our laboratory (Gharbi *et al.* 2002,Chan *et al.* 2005) allowed additional comparison between mRNA and protein expression in the HB4a and C3.6 cell lines. As discussed in Chapter 4, some genes showed similar patterns of protein and mRNA expression in the immunoblotting experiments and in the parallel proteomics experiments. However, for the remaining analysed genes the correlation between mRNA and protein immunoblotting data was generally poor, particularly in growth factor treated samples (Figure 4.20). Thus, the relationship between assessed mRNA levels and the concentration of the respective proteins is much more complex and less clear.

There are several factors to consider that may be responsible for this discrepancy. Firstly, changes in mRNA expression levels do not necessarily translate into changes in protein levels; mRNAs may be unstable and be degraded, or remain untranslated. Secondly, newly synthesized protein may be rapidly degraded, secreted or post-translationally modified so that they are no longer recognized by antibodies (Gygi *et al.* 1999). In addition, the sensitivity and dynamic range of the methodology used to determine protein expression (immunoblotting) may give differences in fold-

change. The specificity of antibodies used in protein expression analysis experiments can also complicate data validation. Indeed, antibody specificity proved to be an important issue in the work carried out here. A number of purchased commercial antibodies, including S100P, annexin II and the transcription factor forkhead box M1 (FOXM1), failed to produce single bands at the correct molecular weight by immunoblotting (data not shown), and therefore expression data for these genes could not be validated. Finally, there is a significant amount of error and noise in both protein and mRNA experiments that limits one's ability to obtain a clear picture regarding the correlation between mRNA and protein expression levels (Chuaqui *et al.* 2002,Greenbaum *et al.* 2003).

In summary, the results presented here show that qRT-PCR is a suitable method for validation of microarray data. Moreover, although it is possible to predict how some proteins are regulated through their gene expression profiles, post-transcriptional regulatory mechanisms appear to play an important role in ErbB-dependent signalling and transformation.

6.3 Cross-talk Between the ErbB and IFN Signalling Pathways

As mentioned above, a number of IFN-stimulated genes (ISGs) were found to be downregulated in the C3.6 cells. IFNs, particularly IFNγ, have been shown to prevent the development of primary and transplanted tumours (Ikeda *et al.* 2002). Some of the mechanisms of action proposed to account for this include (a) inhibition of tumour growth and/or survival, (b) inhibition of angiogenesis, (c) effects on both the innate and adaptive immune response against tumours. Importantly, other microarray studies of breast cancer have identified IFN-regulated genes in their analyses. Perou *et al* showed that in human mammary epithelial cells subjected to 100% confluency or cellular senescence, a number of IFN-regulated genes were induced and clustered together (Perou *et al.* 1999). Furthermore, the response of this set of genes to IFNα and IFNγ stimulation was very similar to the genes' response to senescence or confluence. Genes in this cluster included OAS1, STAT1, GBP1 and IFITM1, all of which were also identified in the present study. A cluster of IFN-regulated genes was also observed in microarray studies using tumour samples from 42 individuals, including 36 infiltrating ductal carcinoma patients (Perou *et al.* 2000), as well as in microarray studies of the NCI60 cancer cell lines, which are derived

from tumours from a variety of tissues and organs (Ross *et al.* 2000), and showed substantial variation in expression among samples. In addition, a recent publication suggested a role for EGFR in the regulation of the IFN signalling pathway, where microarray analysis showed that a number of ISGs (mainly IFN type II-inducible) were induced by EGF in cells overexpressing EGFR, but not in cells that expressed the constitutively active variant EGFRvIII, and this correlated with activation of STAT1 and STAT3 (Pedersen *et al.* 2005). These results, together with the findings presented here, suggest that IFN-related genes are co-regulated in human cancers and play an important role in breast cancer development and progression.

While studying the effects of ErbB2 overexpression on the IFN signalling pathways, we chose to focus on p48 since it is an essential component of the ISGF3 transcription factor responsible for the induction of many type I IFN-inducible genes (Figure 5.1 and (Bluyssen *et al.* 1996)). Indeed, many of the downregulated ISGs were genes known to be responsive to type I IFN (Figure 5.2), suggesting that downregulation of these genes in the C3.6 cells is a consequence of the lower levels of p48 observed in these cells.

Firstly, the downregulation of p48 was confirmed by qRT-PCR as well as immunoblotting, indicating that the gene expression changes are reflected at the protein level. Importantly, an inverse correlation between p48 and ErbB2 protein levels was found in a panel of breast tumour cell lines as well as in a number of HB4a-derived clones expressing ErbB2 (Figure 5.3). In order to establish a causal link between ErbB2 overexpression and p48 downregulation, MCF-7 cells were transiently transfected with the ErbB2 gene. These experiments revealed that the transfection process triggered a response in the IFN pathway, as shown by the increased levels of STAT1 phosphorylation and induction of p48 in the transfected cells. This made the analysis of ErbB2-dependent regulation of p48 expression difficult. Nonetheless, cells transfected with an ErbB2-containing vector generally had reduced p48 protein expression compared with cells transfected with the empty vector, and this was achieved in three separate experiments. Although additional experiments need to be carried out to obtain a definitive conclusion on the downregulation of p48 by ErbB2, the experiments shown here indicate that indeed p48 expression can be negatively regulated by ErbB2.

The remainder of the research into IFN signalling focused on elucidating the mechanistic links between ErbB2 overexpression and the downregulation of p48. It

was found that ErbB2 overexpression does affect the cellular response to IFNs, as shown by the ability of both type I and II IFNs to inhibit the cellular proliferation and to induce STAT1 phosphorylation and the expression of p48 in the C3.6 cells. However, an important finding was that growth factor treatment (EGF or HRG) could block the ability of IFNγ to induce the expression of p48 in the C3.6 cells in an ErbB-dependent manner. Moreover, IFNβ was unable to inhibit growth factor-mediated proliferation in the C3.6 cells. These findings suggest that, in ErbB2 overexpressing cells, activation of ErbB receptors by growth factors results in the inability of IFNγ to induce the expression of p48, which in turn prevents the inhibition of growth factor-induced proliferation by IFNβ. As discussed in Chapter 5, further experiments showed that these cellular effects were not due to differential autocrine/paracrine signalling, cellular localization, protein complex formation or differential post-translational modifications of p48 or STAT1 in the C3.6 cells. Immunoblotting studies using the proteasome inhibitor PS341 suggested a possible mode of regulation of p48 turnover. In addition, the results from time-course experiments with PS341, where p48 levels decreased, indicated that there may be other as yet unidentified proteins involved in the regulation of p48 expression and turnover.

Figure 6.1-a summarizes the currently known mechanisms of regulation of ISGs and of p48. There are two main routes for the transcriptional activation of ISGs: i) genes that are mainly induced by type I IFNs through the ISGF3 transcription factor, which includes p48, binding to the ISRE sequence and ii) genes whose transcription is induced by IFNγ by activated STAT1 homodimers binding to the GAS sequence. The transcriptional activation of p48 is thought to be primarily dependent on IFNγ-induced STAT1 homodimerization and binding to another promoter element (GATE) different from that of other ISGs. Two proteins, termed IFNγ-inducible factors (GIFs), have been reported to bind GATE and to be an essential requirement for p48 transcription (Weihua *et al.* 1997a). The transcription factor CCAAT/enhancer-binding protein beta (C/EBP-beta) was later identified as one of the GIFs (Xiao *et al.* 2001), but the other GIF remains uncharacterized. IFNβ was also able to induce the expression of p48 in the cell lines studied here. The results presented here show that, in ErbB2 overexpressing cells, a number of type I IFN-inducible ISG, as well as p48, are downregulated by ErbB2 overexpression. It is

not known at this stage whether the downregulation of such ISGs is a result of transcriptional inhibition or of post-transcriptional events or both. In addition to being downregulated in C3.6 cells, the increase in p48 expression induced by IFNγ is inhibited by growth factor co-stimulation (Figure 6.1-a). These findings clearly implicate the ErbB signalling network in the regulation of p48 and of the IFN signalling pathway and may have important implications in the development of breast cancer.

Based on the results presented in this thesis, a number of potential mechanisms of p48 regulation are proposed (Figure 6.1-b). As discussed previously, it is likely that the proteasome plays a role in the regulation of p48 levels. However, as inhibition of the proteasome results in downregulation of p48, it is likely that p48 is not itself a target of proteasome-dependent degradation and is degraded through non-proteasomal dependent pathways, such as in lysosomes. Alternatively, p48 may be secreted from cells or re-localized to an insoluble compartment, in which case it would not be amenable to western blotting, although immunofluorescence showed only nuclear staining when it was expressed. A third possibility would involve the existence of an as yet undiscovered protein inhibitor of GIFs or p48 transcription directly that may itself be targeted for proteasomal degradation. Such a GIF inhibitor could also be regulated by growth factors, and this could explain the delay observed when growth factors inhibit IFNγ-mediated p48 induction. It should be noted, however, that the IFN signalling pathway is a highly complex cascade of events which include feedback loops and a number of other highly regulated protein activities, such as those of JAKs and SOCS, which are important in the regulation of signalling capacity through this pathway and could also be regulators of p48 expression and function.

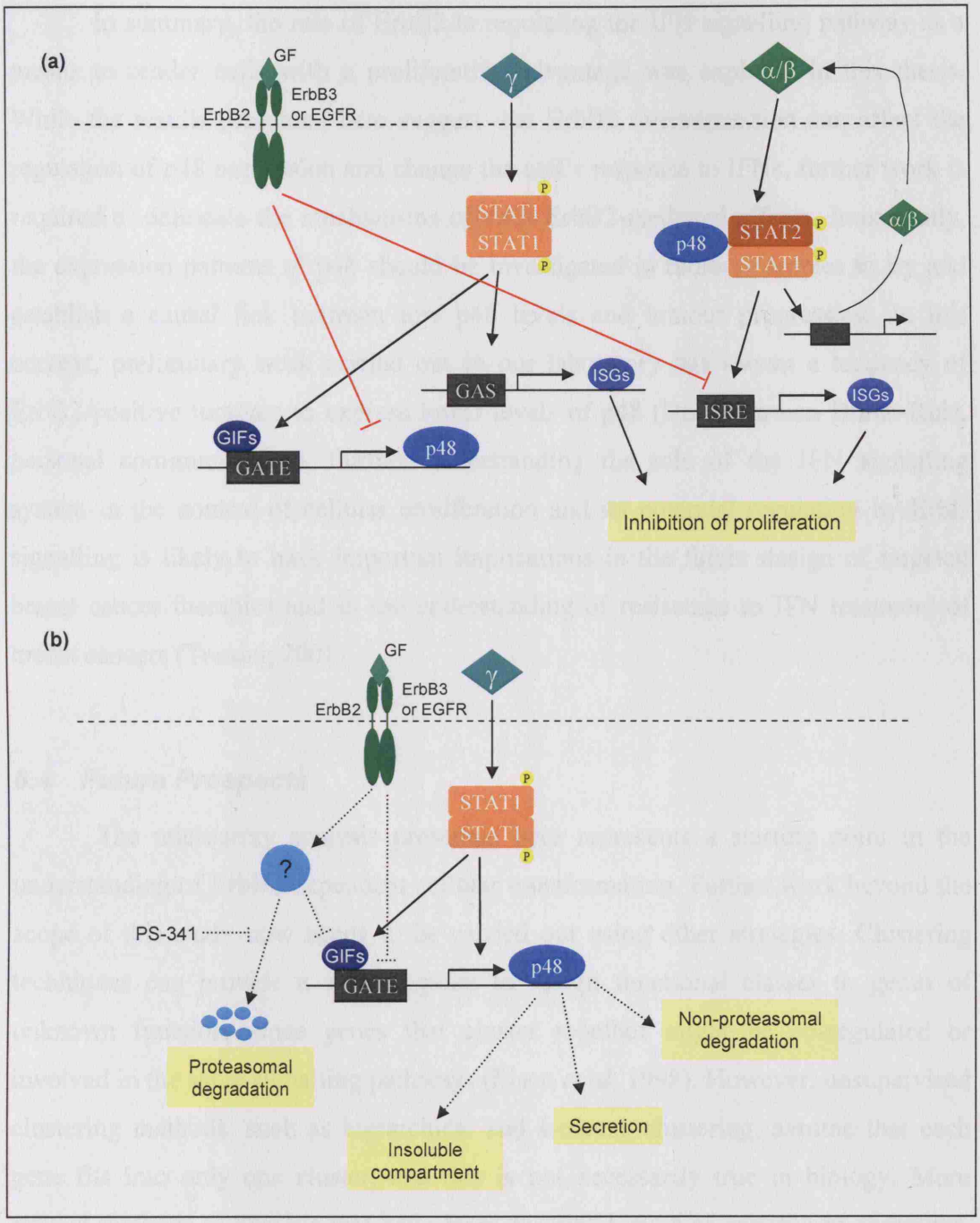

Figure 6.1: Proposed mechanisms of p48 regulation. (a) Model of type I and type II IFN signalling and the effect of growth factor stimulation on p48 expression levels in ErbB2 overexpressing cells. **(b)** A number of hypotheses are presented regarding the possible fate of p48 following transcriptional activation by IFNγ and the role of the proteasome in p48 regulation.

In summary, the role of ErbB2 in regulating the IFN signalling pathway as a means to render cells with a proliferative advantage was explored in this thesis. While the results presented here suggest that ErbB2 overexpression can affect the regulation of p48 expression and change the cell's response to IFNs, further work is required to delineate the mechanisms of such ErbB2-mediated effects. Importantly, the expression patterns of p48 should be investigated in tumour samples to try and establish a causal link between low p48 levels and tumour progression. In this context, preliminary work carried out in our laboratory has shown a tendency of ErbB2-positive tumours to express lower levels of p48 (Dr. MCarmen Duran-Ruiz, personal communication). Further understanding the role of the IFN signalling system in the control of cellular proliferation and its potential regulation by ErbB signalling is likely to have important implications in the future design of targeted breast cancer therapies and in the understanding of resistance to IFN treatment of breast cancers (Tossing 2001).

6.4 Future Prospects

The microarray analysis presented here represents a starting point in the understanding of ErbB2-dependent cellular transformation. Further work beyond the scope of this study now needs to be carried out using other strategies. Clustering techniques can provide a starting point to assign functional classes to genes of unknown function, since genes that cluster together might be co-regulated or involved in the same signalling pathways (Eisen *et al.* 1998). However, unsupervised clustering methods, such as hierarchical and *k*-means clustering, assume that each gene fits into only one cluster, and this is not necessarily true in biology. More refined methods to achieve this have been described, such as supervised computer-learning methods able to recognize genes that are similar in expression pattern to groups of genes known to be co-regulated, building on existing knowledge of gene function (Brown *et al.* 2000). Genes are regulated through the coordinated action of multiple transcription factors. Because of this combinatorial nature, further analysis of microarray data using a pathway perspective could lead to a higher level of understanding of network organization in the ErbB signalling system. For example, by analyzing the promoters of groups of genes and associating the occurrence of transcription factor binding sites with their expression profile, it is possible to link

gene expression data with regulatory processes (Pilpel *et al.* 2001). Several public and commercial pathway resources currently exist that allow researchers to map expression data onto pathways and examples include GenMAPP (Dahlquist *et al.* 2002) and Pathway Processor (Grosu *et al.* 2002).

Further validation of differentially expressed genes is also necessary. Here, only a small percentage of the genes whose expression was significantly changing were analysed further by qRT-PCR. While the majority of results were qualitatively accurate and reflected the patterns of expression obtained by microarray analysis, these were only performed in duplicates. Additional reaction plates should be run to confirm the findings, and samples from a new batch of cells used. Furthermore, ErbB2- and growth factor-dependent changes in gene expression should be analysed in other cell systems to further confirm these findings. Ideally, all genes identified should be further validated using an alternative assay to measure gene expression. However, due to the large number of genes typically identified by microarray analysis this is costly and time consuming. In this regard, medium-throughput qRT-PCR technologies are under development. Preliminary work is currently being carried out in our laboratory using a 384-well MicroFluidic Card (Applied Biosystems), in which it is possible to simultaneously assay the expression of 96 genes in quadruplicate using minimal amounts of cDNA (generally 100 to 500 ng per card) (Abruzzo *et al.* 2005). This method should enable the faster validation of a larger number of genes, although the customization of these cards is expensive.

Most of the characterisation work carried out here was performed on the endogenous proteins and using transfected constructs which overexpress epitope-tagged versions of target proteins (such as the myc-tag) would greatly improve the specificity of the analysis allowing assessment of the roles of target genes in the regulation of the transforming capabilities of ErbB2 overexpression. As an inverse strategy, gene silencing to alter the expression of specific genes of interest, using double stranded interference RNAs (Downward 2004), could be usefully applied in our model cell system. A novel finding in this work was the indication of potential post-translational modifications of p48. It would be interesting to pursue this line of research in more detail, perhaps using overexpressed p48, followed by mass spectrometry analysis to identify potential modification sites. A number of hypotheses regarding the regulation of p48 were presented here, although further testing and additional experiments are required to confirm these. The challenge,

however, will be to integrate all pieces of information into a coherent model of growth regulation in a physiological context relevant to the diagnosis and treatment of breast cancer.

6.5 Conclusions

The work presented in this thesis has combined two genomic technologies as tools in the identification of targets of ErbB2 overexpression and of ErbB receptor-mediated signalling, and used the findings from these analyses to further characterize a set of genes in relation to the biochemical pathways leading to ErbB2-dependent transformation. Initially, microarrays were used to examine large numbers of potential targets, and statistical analysis was used to identify genes that were altered significantly by the conditions examined. Then, qRT-PCR was employed to validate microarray results for a smaller set of genes. Finally, specific genes of interest involved in the IFN signalling pathway were selected for further characterization and cross-talk between the ErbB and IFN signalling pathways was proposed.

Taken together, microarray analysis and follow up experiments have revealed the importance of several subsets of genes which could play important roles in the development of ErbB2-dependent breast cancers. Microarray experiments have already provided a wealth of information on the transcriptional events associated with breast cancer and have been used successfully to classify tumours and identify novel biomarkers associated with this disease. The results presented here provide yet another step towards the understanding of such transcriptional changes and may help to decipher the complex signalling circuitry involved in the proliferation, survival and altered adhesion of breast cancer cells.

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Appendix 1: **List of all genes significantly changing.** Significant genes changing in one or more conditions (ErbB2 and/or growth factor stimulation) were identified by SAM software using pair-wise comparisons between samples. All gene lists were then put together to obtain a total of 775 significantly changing genes. Genes responsive to each condition were then separated according to responsiveness in a Venn diagram (see Figure 3.9) and new gene lists were made for each area within the diagram. These lists are shown below (in alphabetical order, including all genes with no common name annotation and clones represented more than once).

List of 775 genes significantly changing in one or more conditions.						
EGF only (164)	**EGF + ErbB2 (35)**	**HRG only (226)**	**HRG + ErbB2 (43)**	**HRG & EGF (76)**	**ErbB2 only (172)**	**All (59)**
123474_A	147097_A	43410_A	136252_A	131362_A	109166_A	110503_A
127677_A	167150_A	42474_A	145972_A	153275_A	113437_A	136876_A
134924_A	188386_A	40763_A	183315_A	180864_A	120387_B	153508_A
137416_A	276818_A	380426_A	204335_A	214227_A	132508_A	166125_A
142916_A	346510_A	380345_B	2070190_B	222155_B	138768_A	241826_A
1590032_A	36006_A	320151_A	323028_A	247402_A	150304_A	248933_A
161110_B	ANXA1;	307636_A	34497_A	257614_A	152202_A	306537_A
1674777_A	ANXA2;	278252_A	41787_A	271284_A	1552526_A	323828_B
1932804_A	AREG;	1902513_E	51927_A	343072_A	1555540_B	346130_B
220430_A	C14orf31;	1283456_A	784820_A	343396_A	167138_A	724356_A
221567_A	CD59;	127856_A	795557_A	36869_A	204334_A	ALDH1A3;
240613_A	CDH3;	124781_A	795557_B	37690_A	208339_B	B4GALT1;
262841_A	CTSC;	32120_A	CDH13;	40847_A	212036_A	B4GALT1;
265370_A	DDX5;	357309_A	COX6C;	469303_A	243497_A	B4GALT1;
271748_A	EMP1;	38728_A	DARS;	48256_A	248261_A	BCAR3;
272743_A	HSD3B1/B2;	148677_A	ERBB2;	743359_A	249115_A	CNN3;
276692_A	IER3;	162211_A	FBLN2;	ABCF2;	249145_A	COTL1;
291885_A	IGFBP3;	154762_B	GPC1;	ACAS2;	249569_A	CSRP1;
292209_A	ITGA2;	309039_A	HIBCH;	AKAP12;	26156_A	DHCR7;
292996_A	ITGB4;	345025_A	HSPB1;	ALDOA;	266558_A	DUSP1;
299682_B	LCP1;	34783_B	ITGA3;	ATF4;	267484_A	DUSP1;
300027_A	MAP2K1;	31881_A	KRT13;	CCT2;	272969_A	DUSP5;
300590_A	MT1K;	40469_A	KRT15;	CSE1L;	278666_A	DUSP5;
309798_A	MUC1;	45542_A	NEDD9;	CSE1L;	282333_A	EFNB2;
310101_B	NCBP2;	382357_A	NUP214;	CSE1L;	296841_A	EMP1;
320716_A	NEDD9;	376548_A	PKM2;	CSNK1E;	302059_A	FKBP4;
321652_A	POLD2;	205185_A	PLAT;	DHCR24;	302516_A	FOXM1;
324210_A	PPP1R14B;	1030933_B	PRODH;	DHCR24;	302953_A	FTH1;
324990_B	PPP4R1;	138141_A	PSCA;	DHCR7;	303144_A	FXR1;
341122_A	PRC1;	278203_A	SGK;	DKC1;	31740_B	GADD45A;
359849_A	SDC1;	183901_A	SLC1A6;	EBNA1BP2;	324101_A	GSTP1;
361086_A	SERF2;	36695_A	SLC20A2;	EIF4A1;	356945_A	IGFBP3;
36562_A	TAP1;	44350_A	SLC26A2;	ENO1;	359705_A	LDHA;

table cont'd

EGF only (164)	EGF + ErbB2 (35)	HRG only (226)	HRG + ErbB2 (43)	HRG & EGF (76)	ErbB2 only (172)	All (59)
381173_B	TSSC3;	39934_A	SLC7A7;	EPPK1;PLEC1;	365366_B	MAP2K3;
430094_A	TYMS;	123232_A	TIMP3;	ETF1;	381948_A	MT1B;
472138_A	ZFP36L2;	37517_A	TIMP3;	GADD45A;	38728_B	MT1K;
472160_A		24593_B	TOP1;	GRN;	428542_A	MT1X;MT1L;MT1G MT1H;MT1F;
501518_A		276798_A	TRIO;	HNRPAB;	471598_A	MT1X;MT1L;MT1G MT1H;MT1F;
723972_A		30563_A	TRIO;	HNRPU;	485774_A	MT1X;MT1L;MT1G MT1H;MT1F;
757765_A		300986_A	UBC;UBB;C20 orf109;	HPCAL1;	49132_A	MT1X;MT1L;MT1G MT1H;MT1F;
770080_A		31929_A	VIM;	HSPA1L;	501814_A	MYC;
774420_A		167078_A	WFDC2;	ITGA3;	50959_A	MYC;
781436_A		324061_A	ZFP36L1;	KRT6D/B;	51458_A	NGFRAP1;
789170_A		46889_A		LAD1;	668680_A	NME1;
823876_A		469971_A		LAD1;	754611_A	PDGFB;
ACAT2;		471062_A		LAD1;	772457_A	PDLIM1;
ACTB;		501604_A		LCN2;	ABCG2;	PHB;
ADRM1;		51305_B		MAT2A;	AGR2;	PKM2;
AMD1;		52933_A		MELK;	AGRN;	PPIF;
ANXA1;		666849_A		MGST3;	AGRN;	PRDX1;
ANXA1;		714259_A		MIF;	AKAP13;	S100P;
ARHGEF2;		727272_A		MYBL2;	AKAP13;	SFN;
ARL6IP;		728268_A		NFKBIA;	AKR1B1;	TAGLN;
ARPC1B;		730640_A		NOL1;	AKR1B1;	TAGLN;
ATP5G2;		753396_A		NOL5A;	ALDH1A1;	TXNIP;
ATP5G2;		768324_A		PFN1;	ALDOC;	TXNIP;
BCAR3;		783721_A		PRC1;	APLP2;	VCL;
C14orf1;		796241_A		PRDX5;	ATP5G1;	ZFP36L1;
C1orf16;		797102_B		PRNP;	ATP5G1;	ZYX;
C20orf16;		813171_A		PSAP;	ATP5G1;	
C6orf48;		813244_A		PTRF;	ATP5G3;	
CAV1;		823590_A		RPL15;	ATP5G3;	
CCT2;		ACAS2;		RPL15;	ATP5L;	
CD59;		ACP5;		RPL15;	ATP6V1F;	
CD59;		AGPS;		SDC4;	BCL6;	
CDC42EP1;		AK3;		SERPINE1;	BLCAP;	
CDH3;		ALDH4A1;		TCOF1;	BST2;	
CLIC1;		ANXA5;		TGM2;	C10orf7;	
CSE1L;		APOBEC3C;		TUBA1;	C1S;	
CSNK1A1;		ARL6IP;		TUBB;	CALM2/1/3;	
CSNK1E;		ARL6IP;		TUBB;	CHEK1;	
CTNNAL1;		ATP5O;		TXNIP;	CLTC;	
CTNNAL1;		ATP6IP1;		UBE2C;	COX6C;	
DDX17;		ATP6V0C;		UBE2C;	COX8;	
DKC1;		ATP6V1F;		VCP;	CPNE3;	
DNM1L;		B4GALT5;		VIL2;	CREG;	

table cont'd

EGF only (164)	EGF + ErbB2 (35)	HRG only (226)	HRG + ErbB2 (43)	HRG & EGF (76)	ErbB2 only (172)	All (59)
DNMT1;		BIRC2;			CSTA;	
EBP;		BMP1;			CTSG;	
EFNB2;		BPAG1;			CTSH;	
EHD1;		CALM2/ 1//3;			CYBA;	
EMP2;		CALM2/ 1//3;			DARS;	
FABP5;		CAV2;			DLAT;	
FHOD1;		CBX4;			DLAT;	
FKBP1A;		CCNI;			DUT;	
FKBP1A;		CD151;			DUT;	
FTL;		CD38;			FBXL11;	
GADD45B;		CDKN1A;			FKBP4;	
GADD45B;		CKS2;			FXR1;	
GARS;		CMAS;			FYN;	
GBP1;		CPNE1;			G1P2;	
GPSN2;		CPNE3;			GMPS;	
GTF3A;		CRIP2;			GSTP1;	
HRMT1L2;		CTNND1;			GTF3A;	
HRMT1L2;		CTRL;PSMB10			HDLBP;	
ID3;		CTSB;			HIBCH;	
ITGB4BP;		DCTN3;			HSBP1;	
ITGB4BP;		DDX17;			IFIT1;	
KRTHA1;		DDX9;			IFITM1;	
LAMA3;		DGKE;			IFITM1;	
LCN2;		DNCL2A;			IFITM2;	
LYN;		E2F4;			IFITM2;	
MAP4K4;		EHD1;			IL6ST;	
MCM5;		EIF2S1;			IMPA2;	
MT1B;		EIF4EBP2;			ISGF3G;	
MT3;		EIF4EBP2;			KDELR2;	
MTHFD2;		EPPK1;PLEC1;			KRT13;	
MYL9;		EPS15;			KRT13;	
NGFRAP1;		ERBB3;			LCP1;	
NHP2L1;		EZH2;			MADH4;	
NME4;		FAT2;			MDH2;	
NNMT;		FOXM1;			MGST1;	
NPAS2;		GALE;			MYO1E;	
PBX1;		GGT1;			NCKAP1;	
PCDH7;		GJA1;			NDUFS1;	
PEA15;		GNAI3;			NDUFS8;	
PFKFB3;		GPC1;			NDUFV1;	
PFN2;		GPX4;			NEDD5;	
PLAT;		GUK1;			NUMA1;	
PLAUR;		GYS1;			OAS1;	
PLXNA2;		HAT1;			OAS1;	
PLXNA2;		HERPUD1;			OAZIN;	

table cont'd

EGF only (164)	EGF + ErbB2 (35)	HRG only (226)	HRG + ErbB2 (43)	HRG & EGF (76)	ErbB2 only (172)	All (59)
PRDX1;		HMGB2;			OGDH;	
PSME3;		HMGB2;			OLR1;	
PTP4A1;		HMGCR;			PBP;	
PTP4A2;		HMGCS1;			PLAT;	
PTPN1;		HNRPH1;			PLD3;	
PTPN1;		HNRPR;			PPIB;	
PYGB;		HPCAL1;			PPP1CC/CA;	
PYGB;		HSD17B1;			PPP1CC/CA;	
PYGL;		HSPA1L;			PRKR;	
S100A11;		HSPB1;			PRPF8;	
S100A11;		IFRD2;HYAL3			RAB1B;	
S100A2;		IGFBP5;			RAB27A;	
S100A4;		IMPDH2;			RAB7;	
S100A6;		LAD1;			RNASEH2A;	
SHMT2;		LAMA3;			RPL17;	
SIAH2;		LCAT;			RPL28;	
SIAT4C;		LIPA;			RPL37A;	
SKB1;		LSS;			RPN1;	
SLC20A1;		LSS;			RPS6KB2;	
SLC2A1;		LY6E;			S100P;	
SOX9;		MARCKS;			SCAMP2;	
stSG89218		MCP;			SCAMP2;	
stSG89235		MKI67;			SERF2;	
stSG89375		MLP;			SERPINH1;	
TIMP1;		MPV17;			SF3B1;	
TIMP1;		MPZL1;			SLPI;	
TMSB10;		MRPL40;			SP110;	
TMSB10;		NCALD;			SRI;	
TNFAIP3;		NDUFA9;			SRP14;	
TOB2;		NDUFS3;			SRP14;	
TRIO;		NPC2;			SSBP1;	
TRIP6;		NPR2;			SSBP1;	
TXN;		NUCB1;			SSFA2;	
TXN;		NUP153;			SSR4;	
TXNL;		OAZIN;			SSR4;	
UBE2D2;		OSIL;SQSTM1			SSR4;	
UBE2N;		PAWR;			SSR4;	
UMPK;		PCBP2;			ST14;	
VASP;		PCCB;			STMN3;	
VEGF;		PCDH7;			stSG89204	
WDR1;		PEPD;			TAP1;	
YWHAH;		PICALM;			TRAM1;	
ZFP36L2;		PKP1;			TRIP12;	
		PLEKHC1;			TRIP13;	
		PLXNA2;			TXNL;	
		POLD4;			UBE2L6;	

table cont'd

EGF only (164)	EGF + ErbB2 (35)	HRG only (226)	HRG + ErbB2 (43)	HRG & EGF (76)	ErbB2 only (172)	All (59)
		POLR2G;			UBL1;	
		PPP2R4;			UBL1;	
		PPP4R1;			UGP2;	
		PQBP1;			USP14;	
		PRAME;			YWHAZ;	
		PRKCL1;				
		PRNP;				
		PRNP;				
		PSIP1;PSIP2;				
		PSMA3;				
		PSMB3;				
		PTK2;				
		QPRT;				
		RNASEH2A;				
		RNF4;				
		RNF4;				
		RPL15;				
		RPL15;				
		SC4MOL;				
		SDCBP;				
		SDHB;				
		SEMA4D;				
		SERPINA6;				
		SFRS10;				
		SFRS3;				
		SFRS5;				
		SFRS7;				
		SLC16A1;				
		SLC26A2;				
		SLC27A2;				
		SLPI;				
		SMC1L1;				
		SNRPA;				
		SNRPD1;				
		SRPK2;				
		SSR1;				
		STARD3;				
		STAT1;				
		STC1;				
		STK15;STK6;				
		stSG89146				
		stSG89300				
		stSG89389				
		stSG89559				
		SYN2;				
		TCEB1;				
		TFAP2B;				

table cont'd

EGF only (164)	EGF + ErbB2 (35)	HRG only (226)	HRG + ErbB2 (43)	HRG & EGF (76)	ErbB2 only (172)	All (59)
		TFF3; TK1; TM7SF2; TOMM34; TPD52L2; TPST2; UBN1; USP5; VARS2;VARS1; VCP; WFDC2; YWHAQ;				